RECEPTORS FOR IMMUNOGLOBULIN G AND COMPLEMENT ON HUMAN EOSINOPHIL LEUCOCYTES

By

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Declaration

I confirm that the work described in this thesis is entirely my own. I had some technical assistance in some experiments described in Chapter IV (Section IV). Certain reagents used were prepared for me by other workers who are clearly acknowledged.

Signed

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SUMMARY

This dissertation is concerned with (1) the identification of surface membrane markers for immunoglobulin G and complement on human eosinophils; (2) how certain pharmacological mediators, which stimulate eosinophil locomotion, influence the expression of these receptors; and (3) the susceptibility of schistosomula coated with antibody and/or complement to killing by human eosinophils, neutrophils and mononuclear leucocytes.

By the rosette technique and immunofluorescence it was shown that human eosinophils and neutrophils bear membrane receptors for rabbit and human IgG and the human complement components, C3b, C3d and C4. There was a significantly reduced percentage of eosinophils bearing receptors for C4 and C3b when patients with eosinophilia of various aetiology were compared to controls. However, no differences were found with IgG receptors.

The ECF-A tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu) and histamine, agents previously shown to be preferentially chemotactic for the eosinophil, markedly enhanced the expression of human eosinophil receptors for C3b. The enhancement appeared to be highly selective for the eosinophil since there was no evidence that C3b receptors on neutrophils or monocytes were altered by these pharmacological agents. The mediators similarly enhanced receptors for C4 but under the same conditions C3d and IgG (Fc) receptors were unaffected. A number of other pharmacological mediators including bradykinin and the prostaglandins

PGE_1, E_2 and $F_{2\alpha}$, had no apparent effect on eosinophil C3b receptors. However, a major histamine catabolite, imidazole-acetic acid, also recognised as an anaphylaxis-associated eosinophilotactic agent, enhanced eosinophil C3b receptors to a degree comparable to that of histamine.

These results suggested that pharmacological mediators of hypersensitivity may regulate certain eosinophil dependent biological reactions and that there may be a direct relationship between the cell surface 'recognition unit' for eosinophil locomotion and some of the membrane receptors which promote the adhesion of eosinophils to opsonized particles.

Studies were also undertaken to determine the susceptibility of schistosomula coated with antibody (Ab) and/or complement (c), to destruction by human eosinophils, neutrophils and mononuclear leucocytes. It was shown that (1) damage to schistosomula in vitro can be mediated by human eosinophils, neutrophils or mononuclear leucocytes in the presence of either 'Ab alone', 'C alone' or 'Ab + C'; (2) the efficiencies of the three experimental systems were Ab + C > C > Ab irrespective of whether effector cells were granulocytes or mononuclear leucocytes; and (3) preferential killing of schistosomula by the human eosinophil, as compared to the neutrophil, was not demonstrable with Ab alone but only when complement was present either alone or in combination with antibody.

These studies indicate that the regulation of various eosinophil-associated biological events may be dependent on surface membrane markers, especially those for complement.

GENERAL INTRODUCTION

In this thesis experiments concerned with the detection of immunoglobulin and complement receptors on the human eosinophil leucocyte membrane and the pharmacological modulation of their expression, particularly by specific eosinophilotactic agents, are described. In addition, the role that human eosinophils may act as killer cells against the larval stage (schistosomula) of Schistosoma mansoni through the presence of their immunoglobulin and complement receptors is evaluated.

As an introduction a brief review of the vast literature relating both to the eosinophil leucocyte and receptors for immunoglobulin and complement on various cell types will be given.

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CHAPTER I - INTRODUCTION

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A. INTRODUCTION

1.0 HISTORICAL BACKGROUND

Probably the first description of the eosinophil leucocyte was that of Wharton Jones (1848) who described the presence of 'coarse granular cells' in the blood of several species including man, but it was not until 1865 that the cell was recognized by Schultze as a distinct entity and given a morphological description. In 1879 Ehrlich observed that the granules had a greater affinity for eosin dyes and he was the first to coin the name 'eosinophil'. Since that time the eosinophil leucocyte has remained a continuous source of interest for many workers and even as early as 1914, when Schwarz summarized the current literature on the eosinophil, he was able to quote 2,758 references.

2.0 THE STRUCTURE OF THE EOSINOPHIL LEUCOCYTE

The human eosinophil is a granulocyte 12-17 μm in diameter with a bilobed and sometimes trilobed nucleus (Zucker-Franklin, 1968) containing the characteristic large refractile granules which stain bright orange with eosin dye.

2.1 The eosinophil nucleus

The nucleus of the eosinophil has lobular configuration in man and an annular one in the mouse and rat. It contains clumps of condensed chromatin distributed against the nuclear membrane with filamentous connections between the nuclear lobes (Zucker-Franklin, 1968). The eosinophil nuclei, like those of other mature granulocytes, do not contain a nucleolus suggesting that major biosynthetic nuclear processes have ceased in the mature cells.

2.2 The eosinophil granule

The large refractile intracytoplasmic granules have a unique structure which many investigators believe may point to an understanding of specialised functions of the cell. They measure 0.5-1.5 μm in their longer diameter and 0.3-1.0 μm in their shorter diameter (Zucker-Franklin, 1968). They have a relatively high density of 1.24 grams/millilitre (West *et al*, 1975). By ultrastructural studies they were shown to be biconvex discs bound by a double-layer membrane and to contain an electron-dense crystalline-like core in the centre parallel to the long axis of the granule (Grey and Biesele, 1955). The central core is surrounded by a less electron-dense matrix. Reversal of electron density pattern of the core and matrix was observed among the granules of an

individual eosinophil obtained from a patient with eosinophilia (Zucker-Franklin, 1974). Earlier workers made similar observations (Ghadially and Parry, 1965) and they have postulated that heterogeneity of the granules may be related to various functions. The granular core is relatively resistant to mechanical trauma and osmotic lysis and was shown to have a high phospholipid content and to contain a major basic protein rich in arginine (Vercauteren, 1950). Miller et al (1966) reported that the crystalline core which appears as parallel lines with regular spacing is a cubic lattice with sides measuring about 30 A in rodents and 40 A in man. The core is probably responsible for the formation of the Charcot-Leyden crystals apparent whenever massive disintegration of the eosinophil occurs, e.g. in the sputum of patients with allergic asthma (Welsh, 1959).

Mature or 'late' eosinophils contain a second type of smaller granule measuring about 0.1-0.5 μm in diameter. These granules are round, homogenous and do not contain a central core. They contain most of the arylsulphatase and acid phosphatase activity associated with the cell (Parmley and Spicer, 1974).

2.3 The eosinophil cytoplasm

The structure of the eosinophil cytoplasm differs from that of other mature granulocytes in containing more numerous mitochondria, a better developed Golgi zone (Goodman et al, 1957) and the presence of free ribosomes and rough endoplasmic reticulum (Hirsch, 1965). These differences are compatible with the more active metabolic

state of the eosinophil.

2.4 The eosinophil cell membrane

Morphologically the eosinophil cell membrane is similar to that of the neutrophil and lymphocyte. Scanning electron microscopic studies have shown that most eosinophils are spherical with some microvilli. Certain cells show membrane deformations such as ridge-like profiles, 'ruffles' or 'blebs' (Polliack and Douglas, 1975). The cell membrane of the eosinophil differs from that of the neutrophil by being more resistant to changes in osmolarity and solvents such as acetone. This property is useful as it forms the basis for one method of direct eosinophil counting (Discombe, 1946; Speirs, 1952).

2.5 The eosinophil antigens

The eosinophil appears to be antigenically distinct from other mature granulocytes. Recent demonstrations that mature eosinophils bear certain cell-specific antigens made it possible to produce specific antisera against these eosinophil antigens. Mahmoud et al (1973), by immunising rabbits with mouse peritoneal eosinophils and absorbing the serum obtained with other leucocytes, produced a highly specific rabbit anti-mouse eosinophil serum. Later, these same workers produced a monospecific rabbit anti-human eosinophil serum (Mahmoud et al, 1974). Gleich et al (1975) could prepare rabbit anti-guinea pig eosinophil serum; they showed, however, that although neutrophils were not directly affected by this anti-eosinophil serum, these cells could absorb out the anti-eosinophil activity. Therefore, it

was concluded that similar antigens were present on both cell types, but in quantitatively greater amounts on the eosinophil.

Other components within the eosinophil also show a degree of cell or species specificity. For example, the major basic protein is antigenically different in the guinea pig and man (Lewis et al, 1976), whilst eosinophil peroxidase can be distinguished from the neutrophil myeloperoxidase on the basis of antigenic properties (Salmon et al, 1970).

3.0 HISTOCHEMICAL STUDIES AND ENZYME ANALYSIS

A number of enzymes are released following the disruption of the eosinophil granules (Archer and Hirsch, 1963). These include acid-phosphatase, β-glucuronidase, acid β-glycerophosphatase, ribonuclease, arylsulphatase, phospholipase and cathepsin.

3.1 Peroxidase

The eosinophil granules contain a peroxidase which is easily recognised histochemically and which remains bound to the insoluble granule residue following disruption. However, recent histochemical and electron microscopic studies have shown that the peroxidase exists mainly in the small homogenous granules and the matrix of the specific granules of rats (Yamada and Yamauchi, 1966), rabbit (Bainton and Farquhar, 1967), guinea pig (Cotran and Litt, 1969) and man (Enomoto and Kitani, 1966). Eosinophil peroxidase differs both chemically and antigenically from the myeloperoxidase of the neutrophil and does not appear to participate in the peroxidase-hydrogen peroxide-halide bactericidal system (Bujak and Root, 1974).

3.2 Arylsulphatase

Arylsulphatase was first identified histochemically within eosinophils, neutrophils and megakaryocytes by Austin and Bischel in 1961. A year later Tanaka et al (1962) showed that the eosinophil contained the highest amounts of arylsulphatase activity in human leucocytes followed by basophils and neutrophils in the ratio of 8:2:1 respectively. These workers also suggested that any disease process which

induces an eosinophilia will consequently give rise to greatly enhanced blood arylsulphatase levels. Eosinophil arylsulphatase was found to inactivate slow reacting substance of anaphylaxis (SRS-A) *in vitro* in a dose- and time-dependent fashion (Wassermann *et al*, 1975) and also the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Bach *et al*, 1975); both recognised as mediators of immediate hypersensitivity reactions. These effects suggest that eosinophil arylsulphatase may play an important role in modulating hypersensitivity reactions.

3.3 The eosinophil major basic protein

About half the granule content of protein consists of a basic material which is insoluble at physiological pH and binds firmly to acid alanine dyes, possibly explaining the characteristic eosinophilic staining of the cells (Behrens and Marti, 1962). Gleich *et al* (1973) and Gleich *et al* (1974) have purified the major basic protein from guinea pigs, rats and human eosinophils. This basic protein accounted for approximately 55% of the total granule protein, had a molecular weight of 11,000 and had a high content of arginine. Examination of the major basic protein for the possible biological activities revealed no peroxidase activity and weak, if any, antihistaminic activity. In contrast to the neutrophil granule basic proteins, the eosinophil major basic protein did not increase vascular permeability or contract the guinea pig ileum, and had little bactericidal activity against a variety of bacterial strains tested. However, it was shown to neutralise heparin to a degree comparable to that of protamine. The major basic protein may be

responsible for the characteristic properties of radio-opacity (Harris et al, 1961) and autofluorescence (Fuerst and Janach, 1965) of the eosinophil leucocyte.

3.4 Phospholipase

Although phospholipase activity can be demonstrated histochemically in the eosinophil granules (Ottolenghi et al, 1967), the data concerning the type of phospholipase are somewhat conflicting at the present time. However, Kater et al (1976) have reported the presence of phospholipase D activity in human eosinophils. Interestingly this enzyme which is common in plants has rarely been identified in a mammalian tissue.

Other enzymes were also described in the eosinophil leucocyte, e.g. plasminogen (Barnhart and Riddle, 1965; Barnhart, 1968) and both kinin-producing and kininase activities (Melmon and Cline, 1967; Melmon and Cline, 1968).

3.5 Prostaglandins

Prostaglandins E_1 and E_2 may be released by the eosinophil during an allergic reaction. Hubscher (1975a, b) has demonstrated the release of a material, termed the eosinophil derived inhibitor (EDI), from eosinophil-rich fractions separated from the peripheral blood of atopic individuals. This material, which was characterised as a prostaglandin, was shown to inhibit the release of histamine by basophils. Hubscher (1975a, b) proposed that significant amounts of prostaglandins, originating from human leucocytes, were synthesized by the eosinophil and that these cells may modulate the allergic response by inhibiting basophil degranulation as a result of local prostaglandin release.

3.6 Trace elements

Eosinophils contain relatively high concentrations of copper as well as other trace elements including zinc, cobalt, magnesium and manganese (McNary, 1960). The presence of zinc may be of importance since it is known to inhibit histamine release from mast cells (Kazimierczak and Maslinski, 1974), to prevent platelet aggregation and their release of serotonin (Chvapi et al, 1975) and to inhibit macrophage migration, phagocytosis and the subsequent metabolic response (Karl et al, 1973).

4.0 OXIDATIVE METABOLISM

The few reports on eosinophil oxidative metabolism suggest that the cell resembles the neutrophil in terms of the metabolic burst accompanying contact with opsonised particles and phagocytes. Although the large and well-developed mitochondria suggest an aerobic mechanism for energy production, there is some evidence suggesting the possibility of an anaerobic metabolic pathway. Thus, of the glucose utilized by the cell, about 90% is converted to lactic acid while only 10% is oxidized to CO_2 or used for glycogen, amino acid or lipid synthesis (Stjernholm et al, 1969).

In all studies where the oxidative metabolic activities of the eosinophil were compared with those of the neutrophil, it was shown that the resting and the stimulated eosinophil has a higher metabolic activity. In these studies several metabolic variables were tested, e.g. hexose monophosphate shunt, Kreb's cycle and hydrogen peroxide production. The greater oxidative response in the eosinophil was shown to be primarily due to increased activity of nicotinamide-adenine dinucleotide phosphate-H oxidase (NADPH-oxidase) of granular origin rather than soluble NADPH-oxidase which is the enzyme known to operate in the neutrophil (Baehner and Johnston, 1971).

However, in spite of the greater ability of the eosinophil to generate H_2O_2, these cells were found to be less efficient in terms of bactericidal ability, i.e. they are less capable of efficiently utilizing the peroxidase-hydrogen peroxide bactericidal system known to participate in intracellular killing of ingested organisms by the neutrophil.

5.0 ORIGIN, KINETICS AND DISTRIBUTION OF THE EOSINOPHIL

It was shown in the rat that, before birth, eosinophil production occurs both in the thymus and lymph nodes (Rytömaa, 1960); whereas in the adult most of the eosinophils are produced in the bone marrow (Ringoen, 1938). The exact precursor of the eosinophil in the bone marrow has not yet been identified with certainty. However, it is believed that the cell arises together with other granulocytes from a multipotential stem cell and proceeds through several stages before entering the bloodstream as a mature eosinophil.

Studies on the kinetics of the rat eosinophil showed that the cell cycle time is 30 hr in normal rats and 9 hr in animals stimulated with Trichinella spiralis (Spry, 1971a). In the same report it was also calculated that the total bone marrow eosinophil transit time was 5.5 days in unstimulated rats as compared to 3.6 days in parasite-stimulated animals. The eosinophil transit time in the blood has been found to be relatively short in comparison with other cells. The blood half life was shown to be $\frac{1}{2}$ hr in the dog (Carper and Hoffman, 1966), 6-10 hr in the rat (Spry, 1971b) and 4.5-5 hr in man (Herion et al, 1970). More recently Dale et al (1976) studied six patients with hypereosinophilia by isolating their eosinophils, labelling them with ^{51}Cr, reinjecting them and measuring the eosinophil specific activity at various intervals. Initially the radioactivity attributed to the eosinophils declined rapidly within the first 3 hr, but it subsequently increased, peaking at 6-24 hr. Thereafter the radiolabelled eosinophils were removed exponentially from the circulation with a half life of 44 hr. Similar

findings were previously reported by Herion et al (1970) and Greenberg and Chikkappa (1971). These observations are very suggestive of eosinophil recirculation.

The tissue life span of the rat eosinophil was calculated as 2-4 days (Cohen et al, 1967) and they are known to be end cells incapable of division (Hirsch, 1965).

Blood eosinophils are probably in transit from the bone marrow to their definitive tissue sites (Hudson, 1962; Archer, 1963). In normal individuals the eosinophil comprises between 1-5% of the total circulating white cell population and an absolute count of greater than 240 cells/ cu.mm is considered abnormal in man (Discombe, 1946). However, several physiological factors may influence circulating eosinophil levels such as diurnal variations (Rytömaa, 1960), the state of nutrition (Opie, 1904) and oxygen tension (Hudson et al, 1967). Rat eosinophils are normally seen in various tissue sites including the skin, lungs, liver, bone marrow, spleen, uterus, vagina and gastro-intestinal tract (Rytömaa, 1960). It was shown that for every circulating eosinophil there are about 200 mature cells in the bone marrow reserve and about 500 in the loose sub-mucosal connective tissues. Therefore, it seems that there is a continuous turnover of eosinophils, with production and maintenance of a reserve pool in the bone marrow and transport via the blood to certain tissue sites. The fate of the eosinophil is not yet clear in either normal or eosinophilic patients, but damaged cells may either be phagocytosed by macrophages or they may be excreted by passage through the gastro-intestinal and respiratory tracts.

B. RECEPTORS FOR COMPLEMENT AND IMMUNOGLOBULINS

The presence of membrane surface receptors for the Fc portion of specific immunoglobulins and certain complement components on a variety of human and animal cells has been described by various workers. These receptors appear to be an integral part of membrane recognition systems through which the cell may respond to various environmental stimuli.

A brief summary of work on both Fc and complement receptors will be given in this chapter to serve as a background for the experiments which are described in subsequent chapters.

1.0 RECEPTORS FOR IMMUNOGLOBULINS - 'Fc RECEPTORS'

Although the presence of receptors which can recognise a certain region within the immunoglobulin molecules on certain cell types has been recognised since 1966 by Uhr and Phillips, the term 'Fc receptor' was first introduced by Paraskevas et al (1972) to describe in distinct molecular entity, present in the cell membrane, which can recognise and react with some region within the Fc portion of certain immunoglobulin classes. In this context Fc receptors were described on various human and animal cell types. They were described on macrophages (Howard and Benacerraf, 1966), monocytes (Huber and Fudenberg, 1968), neutrophils (Lay and Nussenzweig, 1968), platelets (Henson, 1969), mast cells (Tigelaar et al, 1971), B lymphocytes (Basten et al, 1972; Paraskevas et al, 1972), activated T lymphocytes (Yoshida and Anderson, 1972), various malignant cell populations including lymphomas and leukaemias (Grey et al, 1972; Shevach et al, 1972;

Jondal and Klein, 1973). More recently Fc receptors were also described on human yolk sac and placental membrane (Moskalewski et al, 1975; Matre et al, 1975).

Although all the Fc receptors on various cell types are operationally similar in that they show marked affinity for sites in the Fc region of certain immunoglobulin classes, the phenomenon has various biological implications. For example, on mast cells and basophils the Fc receptors show high affinity for IgE of the same or very closely related species. Similarly, macrophages have high affinity receptors for IgG. Thus the Fc receptors on these cell types will bind to the immunoglobulins even in the absence of an antigen. In contrast, the receptors for IgG on neutrophils, B lymphocytes and platelets are known to be of low affinity and in these cell types it is, therefore, essential that the Fc region is present in the form of an antigen-antibody complex to allow a stable binding to the receptor. Other differences also occur between Fc receptors on various cell types. For example, although it is now agreed that most Fc receptors will bind to specific sites on the $C\gamma_3$ domain of the Fc region, recent studies on the Fc receptors of human placental tissue and of some %K-cells° suggest that both $C\gamma_2$ and $C\gamma_3$ domains may be involved (Yasmeen et al, 1973; Yasmeen et al, 1976).

The reaction between Fc receptor-bearing cells and the Fc portion of the immunoglobulin in the indicator system is considerably stabilised by procedures which alter the conformational structure of the Fc portion, i.e. involvement of the molecule in an antigen-antibody complex formation or

chemical or heat aggregation of the immunoglobulin molecule. Therefore, all techniques used to demonstrate the presence of the Fc receptors have involved the activation of the Fc region leading to conformational changes to facilitate the interactions with the Fc receptor. The most commonly used techniques to detect these receptors on various cell types are:

(a) The Rosette Technique - where erythrocytes are sensitized with a sub-agglutinating titre of IgG (or any other antibody class) preparation with specificity for the erythrocyte antigen. When the sensitized erythrocytes are incubated with the Fc receptor-bearing cell at the optimal conditions clusters are formed in which the receptor-bearing cell is either partially or totally surrounded by red cells. These clusters are usually termed EA 'erythrocyte-antibody' rosettes.

(b) Immunofluorescence - where the immunoglobulin molecule is aggregated either by heat or by chemical agents. The aggregated immunoglobulin is then incubated with the receptor-bearing cells under optimal conditions and binding can be detected by using fluorescent labelled anti-immunoglobulin.

(c) Autoradiography - where the immunoglobulin molecule is labelled with radioactive iodine and either heat aggregated or combined to a specific antigen to form a radioactive antigen-antibody complex, which is then incubated with the target cells and the preparation can be examined autoradiographically to assess the amount of radioactive uptake.

1.1 Specificity of Fc receptors

Apart from the receptor for IgE on mast cells and

basophils, most reports on Fc receptors on various cell types refer to IgG. However, receptors for other immunoglobulins have recently been reported. Capron et al (1975) described the killing of schistosomula of Schistosoma mansoni by rat macrophages in an IgE-dependent system indicating the presence of a receptor for the Fc portion of the IgE class on macrophages. Also Gonzalez-Molina et al (1977) described the presence of a receptor for the Fc region of IgE on a subpopulation of normal human peripheral B lymphocytes. Over the last few years the presence of a receptor for IgM on certain subpopulations of human T and B lymphocytes has also been reported by various workers. Moretta et al (1975) described the presence of IgM receptors on normal human T lymphocytes. This finding was confirmed by McConnell and Hurd (1976) and by Gmelig-Meyling et al (1976). Ferrarini et al (1976) confirmed these observations and showed that the receptor is specific for the Fc portion of IgM. Later Pichler and Knapp (1977) described the presence of a similar receptor on chronic lymphatic leukaemia cells and suggested that this receptor may be a unique property to neoplastic cells since they were unable to detect it on normal B lymphocytes. However, this assertion was not supported by Ferrarini et al (1977) who demonstrated the presence of IgM receptors on normal human B lymphocytes after overnight culture in an IgM-free medium.

1.2 Physiochemical characterization of Fc receptors

Several investigators have attempted to characterize Fc receptors chemically. One approach has been the use of enzymes of known substrate specificity. Using this technique

Davey and Asherson (1967) showed that Fc receptors on guinea pig and rabbit macrophages were resistant to trypsin, chymotrypsin and papain. In fact, several other studies suggested that enzyme treated cells may have a greater affinity to bind antibody than untreated control cells (Arend and Mannik, 1973). However, the same Fc receptors which were found to be resistant to proteolytic enzymes were shown to be susceptible to phospholipase. This does not necessarily imply that they are phospholipid in nature since any perturbation of the fluid bilayer may alter the activity of the constituent membrane proteins. On the other hand, Unkeless and Eisen (1975) have found that the Fc receptor activity of mouse macrophages was lost following a 15 min incubation with trypsin. However, about 60% of the binding activity was restored when the trypsin treated cells were incubated for 12 hr in serum-free medium. More recently, Anderson and Grey (1977) have assayed detergent cell lysates and culture supernatant fluid of mouse cells for the presence of soluble Fc receptors by means of a radiological bioassay. In this study the soluble Fc receptors of all cells tested, except mouse macrophages, behaved as lipoprotein. In the absence of detergent these Fc receptors sedimented as a 20 S macromolecule and as a 7-9 S material in the presence of a detergent.

Therefore, in spite of the rapid development of techniques for isolation and characterization of membrane associated molecules, there remains a considerable controversy concerning the exact physiochemical nature of Fc receptors.

1.3 Biological functions of Fc receptors

The presence of Fc receptors on a variety of cell types

was shown to be a basic requirement for their mediation of several important immunological processes. Some of these processes are reviewed in the following paragraphs.

(i) Phagocytosis

This is one of the major immunological processes in which the participation of Fc receptors is thought to be a vital factor. For the neutrophil it was shown that the IgG coating of sheep red blood cells or S. aureus is an important prerequisite for triggering the internalisation phase of phagocytosis. It is also known that the adherence of the IgG coated particle to the phagocytic cell through its Fc receptor may be the first step towards the completion of the process of phagocytosis.

For the monocyte the presence of the Fc receptor is also important for phagocytosis. However, unlike the neutrophil, the monocyte can ingest target cells without being coated with antibody, i.e. the recognition of an antibody is not an absolute requirement for phagocytosis. There are certainly some other recognition mechanisms whereby the monocyte can recognise the particle as foreign. Although the antibody is not an obligatory requirement for the phagocytosis by monocytes, its presence can greatly enhance the process, presumably by causing close contact between the cell and antibody-coated particle.

(ii) Antibody-dependent cell-mediated cytotoxicity (K-cell cytotoxicity)

In the original description of the killer cell (K-cell) system the effector cells are identified as normal 'non-sensitized non-adherent mononuclear cells which had neither

B-cell nor T-cell characteristics and they were, therefore, thought to belong to the 'null' cell subpopulation of lymphocytes. However, when this phenomenon was studied more closely it became apparent that other cell types including macrophages and polymorphonuclear cells can act as K-cells against various antibody coated target cells. All cells capable of mediating K-cell cytotoxicity are known to bear Fc receptors. The involvement of the Fc receptors was clearly demonstrated by the finding that antibody-dependent damage of target cells can be completely inhibited by blockade of these receptors following the incubation of the effector cells with antigen-antibody complexes. The role that the Fc receptor may play in the induction of the K-cell lysis may either be:

(a) A passive role - whereby the Fc receptor may serve to concentrate the antibody coated target particles in the vicinity of the K-cell and hence facilitating contact between the membranes of the target and the effector cells. The lysis of the target cell may then occur as a result of the action of a membrane-bound effector molecule (a protease or a phospholipase) or the release of a soluble mediator.

(b) An active role - whereby the occupation of the Fc receptor may actively trigger the release and activation of certain effector molecules via a second messenger, e.g. cyclic GMP.

At the moment the precise role of Fc receptors in the K-cell phenomenon is not clear and until the whole molecular mechanism of K-cell lysis is clarified, the role

of Fc receptors in antibody-dependent damage of target cells remains speculative.

(iii) Secretion

The role that Fc receptors may play in inducing the secretion of certain enzymes is best discussed in relation to the macrophage. This cell is known to be a secretory cell for a variety of macromolecular products, e.g. lysozyme and several neutral proteases. Lysozyme is secreted continuously by the macrophage whereas the other enzymes are secreted in association with the stimulation of the cell and with phagocytosis. The involvement of Fc receptors in the secretion process stimulated by antigen-antibody complexes or heat aggregated IgG is probably similar to their involvement in phagocytosis.

The role of Fc receptors on the polymorphonuclear cells in inducing secretion of lysozomal enzymes is thought to be similar to their role in the macrophage.

(iv) The role of Fc receptors on lymphocytes

The presence of Fc receptors for IgG and IgM on various lymphocyte subpopulations will allow these cells to react with immune complexes. The interaction of immune complexes with lymphocytes through their Fc receptors has been shown to induce a variety of important immunological phenomena, including immune regulation by feedback suppression of antibody formation (Sinclair and Chan, 1971), immunological tolerance (Diener and Feldmann, 1970) and immunological enhancement (Sjogren et al, 1971; Baldwin et al, 1972) (reviewed by Kerbel and Davies, 1974).

2.0 COMPLEMENT RECEPTORS

The first indication of the presence of membrane receptors for altered complement components on certain mammalian cells was the demonstration of the adherence reaction between trypanosomes and human erythrocytes in the presence of fresh immune serum (Duke and Wallace, 1930). The adherence of bound C3 to receptors on primate erythrocytes was originally called 'immune adherence' (Nelson, 1953). Subsequently it became apparent that complement receptors had a widespread distribution among various haemopoietic cells. Thus apart from C3b receptors, receptors for C1q, C4 and C3d have also been described.

2.1 C1q receptors

The presence of this receptor was reported on both T and B lymphocytes (Dickler and Kunkel, 1972) and on certain lymphoblastoid cells (Bokisch and Sobel, 1974). At present the biological significance of this receptor is not clear although the release of vaso-active amines by platelets (which may also have a receptor for C1q) can be inhibited by C1q (Suba and Csako, 1976).

2.2 C4b receptors

The occurrence of C4b receptors on human erythrocytes and C4b dependent immune adherence was first described by Cooper (1969). The C4b receptor was shown to react with both cell bound and fluid phase C4b. Later, Bokisch and Sobel (1974) described the presence of a C4b receptor on human B lymphocytes and lymphoblastoid cells. At the same time Ross and Polley (1974) independently demonstrated the

occurrence of C4b receptors on human granulocytes and lymphocytes.

The relationship between the C4b and C3d receptors on human B lymphocytes was evaluated by Bokisch and Sobel (1974) and also by Ross and Polley (1975). Results from inhibitory experiments by both groups favoured the idea that human B lymphocytes have only one receptor for C3b and C4b. However, in experiments performed with several human lymphoblastoid cell lines (Bokisch and Sobel, 1974) evidence was provided for a C3b receptor distinct from the C4b receptor. Recent immunochemical studies revealed a striking similarity between C3 and C4. In the course of complement activation C3 and C4 are cleaved by the enzymes, C3 convertase and C1 esterase respectively, into a low molecular weight 'a' fragment and a large molecular weight 'b' fragment. Upon cleavage C3b and C4b require two binding sites, a labile binding site which allows the molecules to be attached to cell membrane surfaces, antigen-antibody complexes and other particles, and a stable binding site which enables both C3b and C4b to react with C3b or C4b receptors on the surface of various cells. Both molecules are susceptible to the enzyme C3b inactivator (KAF) and so both molecules are rendered 'immune adherence' negative with human erythrocytes.

It is possible that the function of the C4b receptor on phagocytic cells may be in the attachment of antigen-antibody complexes to appropriate cells and hence phagocytosis can be 'triggered'. The reduced clearance of immune complexes in C4-deficient guinea pigs as compared to normal animals may demonstrate the contribution of the C4b receptor on phagocytic

cells to this mechanism. Ross and Polley (1974) have shown that relatively small amounts of C4 were needed for rosette formation with human neutrophils compared with monocytes. This bound C4 greatly enhances the phagocytosis of sheep red blood cells sensitized with IgG by the human neutrophil. The same workers have also suggested that the C4b receptor activity may be more significant than that of the C3b receptor *in vivo*, since complex-bound C4, but not bound C3b, is protected from the action of C3b inactivator by the presence of C2 (Cooper, 1975).

2.3 C3b receptors

The demonstration of the phenomenon of immune adherence (Nelson, 1953) of C3 coated particles to human erythrocytes was the first indication that there is a receptor on certain cell types that is capable of binding the activated third component of complement (C3b). Subsequently this receptor was shown to be widely distributed among both human and animal cells. For example, it was shown to be present on the surface membrane of primate red cells, platelets, macrophages, monocytes, polymorphonuclear leucocytes, T and B lymphocytes, lymphoblastoid cells and glomerular epithelial cells.

These receptors for C3b represent one of the most important ways through which the complement system may be involved in various immunological tissue reactions, since it is known that the activation of the C3 molecule by either the classical pathway C3 convertase or by the alternate pathway to generate C3b is the main step in the complement cascade.

2.3 (a) Physiochemical characterization of C3b receptors

Several workers, using different techniques, have attempted to characterize chemically the C3b receptors. The expression of the C3b receptor on human erythrocytes, lymphocytes and cultured lymphoblastoid cells is sensitive to the enzyme, trypsin, and reducing agents such as dithiothreitol, 2-mercaptoethanol, 2-amino-ethylisothiouronium bromide hydrobromide (AET), reduced glutathione and L-cysteine (Deirich et al, 1974a). The sensitivity of the C3b receptor to these agents was shown to be temperature, time, dose and pH dependent. Raji cells which had lost their C3b receptor activity due to AET treatment will re-express this receptor function after a lag period of 8-10 hr. It was concluded, from these findings, that the C3b receptor is a protein-like structure requiring disulphide bridges for the expression of biological activity.

In 1975 Dierich et al were able to isolate C3b receptor carrying plasma membrane fragments and partially characterized them chemically. The membrane fragments, which were solubilized using detergents, were completely inactive, whereas the C3b receptor activity was still detectable on membrane fragments which were extracted by potassium bromide.

Henson and Neshyba (1976) similarly solubilized platelet membranes in detergents. When this material was fractionated and the individual fractions were tested for inhibition of histamine release from platelets using C3b coated zymosan particles, the inhibitory activity was detected in a number of fractions representing different molecular weights. However, an inhibitory fraction of molecular weight

30-40 x 10^3 daltons was consistently found and this fraction could also bind to C3b coated zymosan particles.

2.3 (b) Biological functions of C3b receptors

The biological functions attributed to the C3b receptor may best be discussed in relation to the individual cell types.

(i) C3b receptors on phagocytic cells

The adherence of complement coated micro-organisms to phagocytic cells is considered to be one of the most important biological functions of the complement system. Thus protection against various micro-organisms is dependent on their opsonization by antibody and C3 and the presence of C3b receptors on the phagocytic cell. The role of complement, and in particular C3, in this respect was clearly demonstrated by the finding that patients with hereditary C3 deficiency suffer from repeated infection although they have normal levels of immunoglobulins and can form antibodies to both thymus dependent and independent antigens. For some time the opsonizing role that C3 may have on various particles was unclear, especially with regard to the part of the C3 molecule involved and the relation between the C3b and Fc receptors on phagocytic cells in initiating the adherence stage prior to the subsequent ingestion. While investigating the functional roles of IgG and C3b in phagocytosis by human neutrophils, Scribner and Fahrney (1976) were able to show that phagocytosis of S. aureus in the presence of heat inactivated serum was severely depressed. They also showed that, in experiments where the particle:neutrophil ratios were varied, the

particle bound C3b could mediate a ten-fold enhancement of the overall phagocytic rate. Also using quantitative data regarding IgG sensitization they were able to show that bound C3b can result in at least a three-fold decrease in the amount of sensitizing IgG needed for 50% maximal neutrophil phagocytic response. More recently Ehlenberger and Nussenzweig (1977) have examined more precisely the role of membrane receptors for C3b and C3d on human phagocytic cells. It was concluded, from their studies, that:

(a) Both C3b and C3d act as opsonins if the phagocytic cell has the appropriate receptors.

(b) C3b and IgG have separate but synergistic roles in phagocytosis.

From these and other findings it is now believed that the role of C3b and possibly C3d receptors in opsonization is mainly in establishing contact between the particle and the phagocytic cell in the adherence phase whereas bound IgG serves as a trigger for the ingestion phase.

Apart from their participation in phagocytosis other roles were suggested for the C3b receptor on monocytes and polymorphonuclear cells. For example, if the C3b coated particle is non-phagocytosible then exocytosis of lysosomal content of the polymorphonuclear cell can occur. Also the interaction between the C3b molecule and the C3b receptor on the neutrophil may cause certain intracellular changes which will promote the cytolytic potential of the cell. With macrophages, the attachment of C3b to the C3b receptor increases their content of lysosomal enzymes within the endoplasmic reticulum and hence enhance their bactericidal

properties. The same signal can promote the production of C3a by macrophages which can act as a specific cytolytic agent for certain tumour cells (Schorlemmer and Allison, 1976).

(ii) C3b receptors on lymphocytes

The role of C3b receptors on lymphocytes has been extensively studied but the results are conflicting. The finding that the interaction between C3b and C3b receptors on lymphocytes has a mitogenic effect (Hartman and Bokisch, 1975) was not confirmed by other workers (Waldmann and Lachmann, 1975; Koopman et al, 1976). A patient with the genetic KAF deficiency, who had continuously high levels of circulating C3b, did not show any lymphocyte changes (Alper et al, 1970).

It was suggested that the C3b receptor on lymphocytes may facilitate the antibody response to antigens. This can be achieved either by promoting bridge formation between the antigen carrying macrophages and lymphocytes or by enhancing the binding of monomeric antigen to the lymphocyte via the C3b receptor.

The interaction between the C3b receptor on the lymphocyte and C3b can induce the release of a mononuclear chemotactic factor leading to the recruitment of the mononuclear cells to the sites of allergic reaction (Koopman et al, 1976; Sandberg et al, 1975).

More important, but perhaps more difficult to ascertain, is the role of complement in the induction of the immune response through the C3b receptor on lymphocytes (reviewed by Pepys, 1976). It was shown that the IgG, IgA and IgE

antibody responses to thymus dependent antigens were suppressed in mice which were depleted of C3 by a purified factor from cobra venom (CVF). The IgM response to thymus independent antigen was unaffected. Various workers have attempted to simulate these situations by using in vitro models (Waldman and Lachmann, 1975; Feldmann and Pepys, 1974), but the results were equivocal. More convincing evidence for the role of the C3b receptor on lymphocytes in the induction of the immune response was provided by the work of Klaus and Humphrey (1977). They showed that since CVF is a potent thymus dependent antigen in mice, the C3 depletion produced following CVF injection is transient and subsequent injections of CVF are neutralised by antibody. However, prolonged C3 depletion can be produced by repeated injection of CVF in mice which fail to produce antibody response. These mice were unable to localise antigens on the surface of the dendritic reticular cells within the lymphoid tissue and, therefore, the formation of germinal centres and the development of B cell memory was not achieved. This suggests that one role of complement in the induction of the immune response is to facilitate the localisation of antigens on the surface of the dendritic cells through their C3b receptors.

(iii) C3b receptors on 'K-cells'

The role of complement receptors in K-cell activity has been studied by several workers (Van Boxel et al, 1974; Perlmann et al, 1969; Perlmann et al, 1974). Perlmann et al (1975) have shown that cell-bound C3b did not trigger cytolysis, but it can potentiate the antibody-dependent

damage of target cells. Preliminary results (Grant, A., unpublished observations) have also indicated that the presence of bound C3b on sensitized sheep red cells can significantly potentiate the cytolytic ability of human lymphocytes. It seems likely, therefore, that the enhancing role of bound C3b on K-cell cytolysis is due to the amplification of the attachment phase which is an essential preliminary step in such reactions.

(iv) C3b receptors on human glomeruli

Gelfand et al (1975) described the presence of a receptor for bound C3b on normal human glomerular tissues. Later the receptor site was located, using scanning electron microscopic studies, on the visceral epithelial cells of the renal glomerulus (Shin et al, 1977). The presence of this receptor may explain the pathogenesis of immune complex deposition in the glomeruli since one of the known features of immune complex glomerulonephritis is the deposition of complement-containing immune complexes in the glomerulus leading to complement-mediated immune damage.

(v) C3b receptor on primate red cells

There is no known erythrocyte function which can be regarded as a direct result of interaction between C3b and C3b receptors on erythrocytes. However, it has been suggested that the presence of such a receptor may be one way by which the ingestion of bacteria coated with antibody and complement can be facilitated since it is thought that their adherence to the C3b receptor on the erythrocyte will hold them against a rigid object and hence they can be more easily ingested (Nelson, 1956).

(vi) C3b receptors on platelets

C3b receptors are found on the platelets of certain species. In contrast, human platelets apparently lack this receptor, although they do bear a receptor for the Fc portion of IgG and it is thought that this receptor may substitute for the lack of C3b receptors on human platelets in certain *in vivo* reactions. A possible role for C3b receptors on platelets has been suggested by Henson (1970) who demonstrated that in the rabbit the release of vaso-active amines from platelets is enhanced following their adherence to C3b coated particles.

2.4 C3d receptors

The presence of this receptor was first detected by Okaka and Nishioka (1973) on lymphoblastoid cell lines. They were then shown to be present on a variety of cells including normal human B lymphocytes (Ross *et al*, 1973; Eden *et al*, 1973) and monocytes. Initially, C3d receptors were thought to be absent in neutrophils (Ross *et al*, 1973; Eden *et al*, 1973) but recently their presence on human neutrophils has been reported and it is thought that their appearance in these cells may be an indication of an earlier stage of cell maturation (Ross *et al*, 1978).

The function of the C3d receptor is thought to be similar to that of the C3b receptor on phagocytic cells, i.e. they enhance the attachment phase of phagocytosis.

C. RECEPTORS FOR IMMUNOGLOBULINS AND COMPLEMENT ON THE EOSINOPHIL LEUCOCYTE

The first evidence that the eosinophil leucocyte may have membrane surface receptors for complement and IgG was provided by Henson (1969) who observed that guinea pig eosinophils contaminating his neutrophil preparations formed rosettes with erythrocytes coated with IgG and also with IgM and complement. The report of Butterworth *et al* (1975), where human eosinophils obtained from normal healthy individuals were shown to be the main effector cells mediating antibody-dependent cell mediated damage of schistosomula of *Schistosoma mansoni*, has further stimulated a number of laboratories to study animal and human eosinophils for the presence of receptors for immunoglobulin and complement. Rabellino and Metcalf (1975) examined cells from mouse eosinophil colonies, grown *in vitro*, for the presence of IgG and complement receptors and compared them with cells from macrophage and neutrophil colonies. They found that in contrast to macrophage and neutrophil preparations, eosinophils showed no detectable C3 receptors. However, after 7 days in culture 50-60% of eosinophils showed receptors for rabbit IgG. Later, Butterworth *et al* (1976) reported that guinea pig peritoneal eosinophils were capable of reacting in a rosette assay with IgG from guinea pig and pig, but not from rabbit.

While studying human eosinophils to compare the structural and functional properties of cells from normal individuals and eosinophilic patients, Tai and Spry (1976) and Spry and Tai (1976) found that eosinophils obtained from

patients with eosinophilia associated with various clinical conditions have receptors for rabbit IgG. However, they were unable to detect the presence of this receptor for rabbit IgG on eosinophils from normal individuals. They also showed that eosinophils from patients with eosinophilia and from healthy individuals can form rosettes with EAC3 to a similar extent. These workers suggested that the presence of a receptor for rabbit IgG on human eosinophils may be an indication of eosinophil stimulation and maturation.

Sher and Glover (1976) reported the presence of receptors for C3 and also for unsensitized sheep red cells on human eosinophils obtained from patients with eosinophilia due to various aetiology.

From their extensive study of human eosinophil surface markers, Gupta et al (1976) concluded that these cells bear receptors for aggregated human IgG and for the complement components C4, C3b and C3d. However, they were unable to detect receptors for either rabbit IgG or for unsensitized sheep red cells. Also, they could not detect the presence of surface immunoglobulins.

More recently Parrillo and Fauci (1978) were unable to detect EA rosettes with human eosinophils obtained from normal individuals, although they were present on cells obtained from eosinophilic patients tested. They were also able to demonstrate the presence of complement receptors on eosinophils from both normal individuals and from patients with eosinophilia.

For some time various workers have attempted to detect

the presence of IgE on the surface membrane on the eosinophil leucocyte. However, Ishizaka et al (1970) were unable to demonstrate the uptake of I-radiolabelled IgE aggregates on the eosinophil whereas they were able to demonstrate such an uptake on to basophils. Similarly, Sullivan et al (1971) who utilised electron microscopy with ferritin-labelled anti-IgE could detect the presence of IgE on basophils but not on eosinophils. However, Hubscher (1975a) reported that some patients with ragweed antigen hypersensitivity have IgE demonstrable on 20-30% of their eosinophils and that these cells are capable of binding ragweed antigen. Also Fujita et al (1975) have shown that eosinophils recovered from the nasal secretions of patients with ragweed allergy have bound and possibly phagocytosed IgE-ragweed antigen complexes.

In summary, it seems that membrane receptors for both complement and immunoglobulin may be present on both human and animal eosinophils, although some controversy still remains. It is possible that differences in techniques used might explain some, if not all, of these discrepancies.

CHAPTER II - THE AIMS OF THE PRESENT STUDY

THE AIMS OF THE PRESENT STUDY

The eosinophil leucocyte was first characterized by Paul Ehrlich in 1879. Since that time this cell has been a source of interest to numerous investigators, but despite thousands of publications the primary function of the eosinophil remained an enigma although very recently several functional roles have been suggested for the cell in relation to helminth diseases and in immediate-type hypersensitivity (reviewed by Kay, 1976).

An increase in the number of eosinophils both in tissues and the peripheral blood is associated with many clinical conditions. They include allergic diseases such as extrinsic bronchial asthma, atopic dermatitis and allergic rhinitis; hypersensitivity to numerous drugs; pulmonary eosinophilia; certain malignant conditions including Hodgkin's disease; a number of connective tissue disorders and the hypereosinophilic syndrome. In terms of world population the commonest associations with eosinophilia are almost certainly helminth diseases.

The functions of the eosinophil in immediate-type hypersensitivity reactions are probably diverse and, therefore, point to the versatility of the cell in various inflammatory states. It may have a regulatory role at all stages of the allergic reaction, namely mediator release, mediator inactivation and mediator replenishment. Tissues containing mast cells can be sensitized by IgE (or other tissue sensitizing antibody) for the antigen-induced release of chemical mediators of anaphylaxis. These pharmacological

agents include histamine, slow reacting substance of anaphylaxis (SRS-A) and an eosinophil leucocyte chemotactic factor of anaphylaxis (ECF-A). ECF-A selectively attracts eosinophils from a mixed leucocyte population *in vitro* (Kay *et al*, 1971). Histamine may also contribute to the infiltration and localization of eosinophils (Clark *et al*, 1975). Following mediator release an eosinophil-derived inhibitor of histamine release (EDI) - probably a prostaglandin - may then inhibit further histamine release (Hubscher, 1975a, b). Eosinophil histaminase (Zeiger and Colten, 1974) and arylsulphatase (Wasserman *et al*, 1975) are then available for histamine and SRS-A inactivation. Following mediator release and inactivation a repair process is initiated which leads to mediator replenishment. The eosinophil may have an important 'negative' or 'dampening' role in the repair process also. Depletion of eosinophils by anti-eosinophil serum (AES) resulted in more rapid histamine accumulation following cutaneous anaphylaxis (Jones and Kay, 1976). Therefore, eosinophils may have a regulatory role in repair following anaphylaxis and so provide a homeostatic mechanism whereby the antigen-induced release of pharmacological mediators of anaphylaxis from the mast cells is not perpetual in situations of continuous antigenic stimulation.

The clear association between eosinophils and parasites has encouraged many investigators to examine both the mechanisms of parasite-induced eosinophilia and the possibility that the eosinophil is an effector cell in parasite destruction; a role previously suggested by Kay (1974).

More recently there have been a number of studies to support this concept. In 1975 Butterworth *et al* reported that the human eosinophil may act as a principal effector cell in an antibody-dependent damage to schistosomula of *Schistosoma mansoni in vitro*. Furthermore, eosinophils from infected mice were shown to destroy the schistosome eggs in a system where the cell became 'armed' by a cytophilic antibody directed against the appropriate egg antigen (James and Colley, 1976). More recently eosinophils were shown to be the effector cells in the killing of the epimastigotes from *Trypanosoma cruzi* (Sanderson *et al*, 1977). Using a technique which depends on the release of RNA from *T. cruzi*, eosinophils, in the presence of specific antibody, mediated killing whereas lymphoid cells had insignificant activity.

From the previously mentioned work it is evident that the eosinophil leucocyte may be involved, as an effective cell, in a number of important immunological reactions, all of which may require effective recognition mechanisms through which contact between the eosinophils and target cells is made possible. In the present study the need to define more precisely the membrane surface receptors for immunoglobulin and complement was largely stimulated by the report of Butterworth *et al* (1975) in which human eosinophils from normal donors were shown to be the principal effector cells in antibody-dependent killing of schistosomula *in vitro*. In this system the antibody was identified as IgG (Butterworth *et al*, 1977b) and the adherence of the eosinophil to IgG-sensitized schistosomula may be dependent on the presence of freely available IgG receptors. Although there was no

apparent requirement for complement in this system, the possibility that complement may enhance the cytotoxic capacity of the eosinophil was not excluded. Therefore, the main objectives set prior to the commencement of the present studies were:

1. To establish more precisely the presence and properties of membrane receptors for IgG and complement on the human eosinophil leucocyte, especially in view of the conflicting literature reports on the surface markers on eosinophils (see Introduction).
2. To compare these receptors with those present on the human neutrophil leucocyte.
3. To study the pharmacological control of the expression of these receptors by mast cell— derived agents known to be specifically chemotactic for eosinophils.
4. To establish an in vitro system for the assessment of the role of the eosinophil as a killer cell against schistosomula of Schistosoma mansoni.
5. To examine the possible relationship between IgG and complement receptors on eosinophils and schistosomula killing by studying the killing mechanism in the presence of antibody and/or complement.
6. To compare the schistosomula killing capacity of human eosinophils and other human leucocytes.

CHAPTER III - MATERIALS AND METHODS

CHAPTER III - CONTENTS

1.0 BUFFERS

1.1 Dextrose-gelatin-Veronal buffer ($DGVB^{++}$)

This was prepared on the day of the experiment by mixing equal volumes of isotonic Veronal buffered saline (containing 0.0015 M Ca^{++}, 0.0005 M Mg^{++} and 0.1% gelatin Veronal buffer (GVB^{++})) with 5% dextrose in water containing the same concentration of Ca^{++} and Mg^{++} ($D5W^{++}$), as described by Nelson et al (1966).

1.2 Gelatin-Veronal buffer (GVB^{--})

This was prepared as for GVB^{++} but without the addition of Mg^{++} and Ca^{++}.

1.3 0.01 M EDTA GVB^{--} and 0.04 M EDTA GVB^{--}

A stock solution of 0.086 M EDTA, pH 7.4, was prepared and kept at 4°C. To prepare 0.04 M EDTA GVB^{--} and 0.01 M EDTA GVB^{--} the stock solution was diluted in GVB^{--}.

1.4 Tyrode's buffer

This was prepared by mixing 40 ml of Tyrode's A (see below) with 1 g of $NaHCO_3$, 1 g D-glucose, 1 ml 20% $CaCl_2$ and 0.4 ml 25% $MgCl_2$ and adjusting the volume to 1 litre with distilled water. The pH was then adjusted to 7.4.

Tyrode's A

A stock solution containing 200 g NaCl, 5 g KCl and 1.625 g $Na_2H_2PO_4$ in 1 litre of distilled water was prepared and stored at 4°C.

2.0 CHEMICALS AND REAGENTS

Reagents were obtained as follows.

2.1 Eosinophil chemotactic factor of anaphylaxis (ECF-A) tetrapeptides

The valyl- (HCl) (Val-Gly-Ser-Glu), molecular weight 427, and alanyl-peptide (HCl) (Ala-Gly-Ser-Glu), molecular weight 497, were a gift from Dr. R. Camble (I.C.I. Limited, Pharmaceuticals Division, Alderley Park, Macclesfield).

2.2 Histamine acid phosphate was purchased from BDH Chemicals, Poole.

2.3 Bradykinin triacetate and 5-hydroxytryptamine were purchased from Sigma, Kingston-upon-Thames.

2.4 Prostaglandins E_1, E_2 and $F_{2\alpha}$ were supplied by Dr. John Pike, Upjohn Company, Kalamazoo, U.S.A.

2.5 Imidazoleacetic acid hydrochloride (ImAA) chromatographically free of histidine, histamine acid isopropylester, 98.8% pure, 1-methyl-4-imidazoleacetic acid hydrochloride (1,4-MeImAA), L-histidine, free base, 1,4-methylhistamine dihydrochloride (1,4-MeHm), N-acetylhistamine (N-AcHm), all chromatographically homogenous, were obtained from Calbiochem Limited, San Diego, California, U.S.A.

2.6 Timothy grass pollen was a generous gift from Beecham Research Laboratories, Betchworth, Surrey.

2.7 Monospecific anti-C3d was purchased from the Netherlands Red Cross Blood Transfusion Service, Amsterdam.

2.8 Anti-human C3 was a generous gift from Professor D.K. Peters, Department of Medicine, Royal Postgraduate Medical School, London.

2.9 Anti-human C4 was kindly supplied by Dr. E.A. Dewar, Department of Pathology, University of Edinburgh.

2.10 Bovine serum albumin was purchased from Miles Laboratories, Slough, and aggregated according to the method of Dickler (1974).

2.11 Fluorescent anti-human C3 and anti-human C4 were purchased from Hoechst (Behring), Middlesex.

2.12 Human C2-deficient serum was kindly supplied by Dr. J.F. Mowbray, St. Mary's Hospital Medical School, London.

3.0 SHEEP RED BLOOD CELLS (E)

3.1 Sheep blood was drawn aseptically into sterile Alsever's solution and kept at 4°C. Immediately before use the red cells were washed twice in cold saline solution (0.15 M NaCl), once in 0.01 M EDTA GVB^{--} and then adjusted to the required concentration, in the same buffer, spectrophotometrically.

3.2 Treatment of sheep red blood cells with aminoethyl-isothiouronium bromide hydrobromide (AET)

This was performed according to the method of Kaplan and Clark (1974). Briefly, AET (Aldrich Laboratories, Milwaukee, Wisconsin, U.S.A.) was prepared freshly as a 0.143 M solution and the pH was adjusted to 9.0. To 1 ml of washed and packed sheep red cells, 4 ml of AET solution were added. The suspension was thoroughly mixed and incubated at 37°C for 15 min with frequent agitation. The cells were centrifuged and the cell pellet was washed four times with cold saline before they were finally adjusted to yield a 10% solution using medium 199/20% FCS. In all the experiments mentioned in this study the AET-treated sheep red cells were used immediately after treatment.

4.0 RABBIT, BABOON AND HUMAN ANTISERA

4.1 Rabbit anti-sheep red blood cells (A)

Multiple injections of sheep red blood cell stroma were administered to rabbits as described by Rapp and Borsos (1970). The IgG and IgM fractions of the antiserum were obtained by gel filtration on Sephadex G-200. Briefly, a 2.6 x 80 cm column was used with 0.05 M tris in 0.15 M NaCl, pH 7.4, as the eluting buffer. Four millilitres of the anti-sheep red cell serum was applied to the column and 2 ml fractions were collected. The active fractions in the first two protein peaks were located, pooled and divided into 1 ml portions and kept at -20°C. The presence of IgG and IgM in each pool was confirmed by immunoelectrophoresis. Finally the optimal haemolysin titre was determined for each pool (Rapp and Borsos, 1970).

4.2 Human immunoglobulin G (IgG)

This was kindly prepared by Dr. S. Moore, Edinburgh Blood Transfusion Service, by diethylaminoethyl (DEAE) cellulose ion exchange chromatography. Its purity was verified by radial immunodiffusion using ultra-low level plates (Hyland).

It was divided into two portions, one of which was used to prepare heat-aggregated material and the other was used as the normal unaggregated control. The heat-aggregated material was prepared according to the method of Dickler and Kunkel (1972) by heating 2.5 ml of the IgG suspension at 63°C for 30 min. The resultant aggregates were homogenized in phosphate buffered saline (PBS) and

finally diluted to give a protein concentration of 1.2 mg/ml.

4.3 Human anti-schistosomula sera

(a) Three batches of human anti-schistosomula serum were used. They were kindly supplied by Dr. D.S. Ridley, Hospital for Tropical Diseases, London, Dr. W.N. Beesley, Liverpool School of Tropical Medicine and Dr. M.O. Gad el Rab, Department of Microbiology, University of Khartoum. All the sera were inactivated for 1 hr at 56°C before being tested separately for their schistosomula-cytotoxic activity. Each batch was then pooled, aliquoted into 1 ml portions and kept at -20°C until use.

(b) Preparation of the IgG fraction from human anti-schistosomula serum

Serum was fractionated by DEAE-cellulose ion exchange chromatography (DE-52) using 0.01 M Tris, 0.002 M EDTA in 0.025 M NaCl (pH 7.5) as the eluent buffer. Three millilitres of serum were applied to 30 ml of DEAE-52 in a small column and 1 ml fractions were collected. The fractions containing the protein peak were pooled and dialysed against distilled water overnight. The IgG was freeze-dried and reconstituted in medium 199 to the original serum volume. The purity was tested by immunodiffusion against low level plates (Hyland). It was found to be free from IgA and IgM. Finally it was aliquoted and kept at -20°C until required.

4.4 Human anti-D sera

The main batch of anti-D serum used in the experiments described here was kindly supplied by Dr. S.J. Urbaniak,

Edinburgh Blood Transfusion Service. It was prepared as follows. Several plasmapheresis packs were obtained from a donor who aborted due to haemolytic disease of the newborn as a result of anti-D immunisation. The plasma was converted to serum by the addition of one drop of thrombin (Parke-Davis) per ml of plasma. After incubation at 37°C for 1 hr, the material was centrifuged and the clear supernatant transferred into clean containers. The serum was then heat inactivated at 56°C for 30 min. The specificity of the anti-D was confirmed by testing against a panel of standardised red cells by conventional saline, enzyme and indirect antiglobulin methods.

Other batches of anti-D sera which were used in some experiments were supplied by Mr. C. Darg, Edinburgh Blood Transfusion Service. They were prepared as described above. All anti-D batches were aliquoted and kept at -40°C until use.

4.5 Baboon anti-schistosomula serum

This was a generous gift from Dr. Anthony E. Butterworth, Robert B. Brigham Hospital, Boston, U.S.A. It was also heat inactivated for 1 hr at 56°C, divided into 1 ml portions and kept at -20°C until use.

5.0 SENSITIZATION OF SHEEP RED BLOOD CELLS

Either IgG or IgM fractions of the rabbit anti-sheep red cells were used.

5.1 Sensitization with IgM anti-sheep red cells

About 5 ml of sheep red cells in Alsever's solution were washed (2,000 rpm for 10 min at 4°C) twice in 0.15 M NaCl, once in 0.01 M EDTA GVB^{--} and the cell concentration was adjusted to 1×10^9 cells/ml in 0.01 M EDTA GVB^{--}. To these calibrated red cells the IgM fraction was added in amounts causing optimal sensitization needed for complement fixation, as calculated by determining the optimal haemolysin titre. In experiments where the presence of receptors for IgM was tested the sensitization was achieved by using the maximum subagglutinating IgM titre. The mixture was incubated at 37°C for 30 min in a shaking water bath, and at 0°C for 30 min with frequent mixing. The cells were then washed once in 0.01 M EDTA GVB^{--} and twice in $DGVB^{++}$. These cells are kept in $DGVB^{++}$ at 4°C (EA_M^{rab}).

5.2 Sensitization with IgG fraction

The procedure was the same as described for IgM except that the titre of IgG was always the maximum subagglutinating titre. After the final wash the sensitized cells were kept in $DGVB^{++}$ at 4°C (EA_G^{rab}).

6.0 PREPARATION OF COMPLEMENT COATED SHEEP RED CELLS

6.1 Human purified complement components

Human C1 was prepared by euglobulin precipitation according to the method of Vroon et al (1970).

Human C4 was prepared by sequential precipitation and ion exchange chromatography on DEAE-Sephadex and carboxy-methyl (CM)-Sephadex. The eluents used for the DEAE-column were (a) 0.05 M Tris (hydroxymethylaminomethene), 0.002 M EDTA, pH 8.0, and (b) 0.05 M Tris, 0.5 M NaCl, 0.002 M EDTA, pH 8.0, and for the CM-Sephadex column were (a) 0.05 M acetate, 0.002 M EDTA, 0.05 M NaCl, pH 5.0 and (b) 0.05 M acetate, 0.002 M EDTA, 0.3 M NaCl, pH 5.0. C4 activity in the different fractions was detected antigenically and haemolytically; fractions showing maximum activity were pooled, concentrated, aliquoted and kept in liquid nitrogen until use. The C4 haemolytic titre was determined according to the method of Rapp and Borsos (1970).

Human C2 was kindly supplied by Dr. K. Whaley, Department of Pathology, Western Infirmary, Glasgow. Two batches of purified human C3 were used, the first was a generous gift from Professor D.K. Peters, Department of Medicine, Royal Postgraduate Medical School, London. The second batch together with human C5, C6, C7, C8 and C9 were purchased from Cordis Laboratories (Miami, Florida, U.S.A.). Purified human C3b inactivator was kindly supplied by Professor P.J. Lachmann, Laboratory of Molecular Biology, University of Cambridge, but in some experiments Cordis C3b inactivator was used. All these complement components were functionally pure

and free of other haemolytically active components as assayed by effective molecular titration as described by Rapp and Borsos (1970).

6.2 For the preparation of complement coated intermediates, sheep red cells sensitized with rabbit IgM (EA_M^{rab}) as described in 4.1 were used.

(a) EA_M^{rab} were mixed with 400 effective molecules of human C1 per red cell. The mixture was incubated for 90 min at 0°C with periodic shaking. It was then washed once in $DGVB^{++}$ and kept as $EA_M^{rab}C1^{hu}$ (EAC1).

(b) $EA_M^{rab}C1^{hu}4^{hu}$ cells were prepared by the addition of 4000 effective molecules of human C4 per EAC1 cell. The mixture was incubated at 37°C for 30 min. The cells were then washed four times in $DGVB^{++}$ and kept as $EA_M^{rab}C1^{hu}4^{hu}$ (EAC14) cells. The presence of bound C4 on sheep red cells was detected by agglutination with anti-human C4, by showing a positive fluorescence with fluorescent anti-human C4 or by detection of red cell lysis following the completion of the complement cascade, i.e. by addition of C2-C9. In the experiments designed to detect C3 rosettes the amount of C4 added per EAC1 was only 400 effective molecules (not sufficient to form C4 rosettes with either eosinophils or neutrophils).

(c) $EA_M^{rab}C1^{hu}4^{hu}2^{hu}$ cells were prepared by the addition of 50 effective molecules of human C2 per EAC14 cell and the mixture was incubated for 10 min at 30°C. The cells were then centrifuged at 2000 rpm at 4°C for 10 min. The supernatant was discarded and the pelleted cells were kept at 0°C as $EA_M^{rab}C1^{hu}4^{hu}2^{hu}$ (EAC142).

(d) $EA_M^{rab}C1^{hu}4^{hu}2^{hu}3^{hu}$ were prepared as follows: 400 effective molecules of human C4 were added to EAC1 and the mixture was incubated for 30 min at 37°C and washed four times in $DGVB^{++}$. To this EAC14 intermediate (which had insufficient bound C4 to form rosettes with either neutrophils or eosinophils), 50 effective molecules of human C2 were added and the mixture was incubated at 30°C for 10 min without washing. To these EAC142 cells, 2500 effective molecules of human C3 were added. The mixture was incubated at 37°C for 30 min and the cells were washed twice in $DGVB^{++}$ and used as $EA_M^{rab}C1^{hu}4^{hu}2^{hu}3^{hu}$ (EAC3b) cells. The presence of bound C3 on the cells was confirmed by agglutination with anti-human C3, by their positive reaction with fluorescent anti-human C3 and by the addition of C5-C9 to detect lysis.

(e) EAC1423d intermediates were prepared by treating 0.5 ml of EAC3b cells containing 1×10^8 cells/ml with 0.5 ml of the C3b inactivator. The mixture was incubated for 1 hr at 37°C. The cells were then washed twice in $DGVB^{++}$ (EAC3d). The presence of the C3d cells was confirmed by testing the C3d cells for immune adherence with human group O Rh positive erythrocytes. In all these experiments 90-95% inhibition of immune adherence was aimed at. On some occasions the addition of C3b inactivator was repeated and a longer incubation time was found to be necessary for complete conversion of the C3b cells to C3d cells. The EAC3d cells, in contrast to the EAC3b cells, were not lysed in the presence of C5-C9. However, they were agglutinated with monospecific anti-C3d, as were the EAC3b cells.

7.0 PREPARATION OF HUMAN EOSINOPHILS, NEUTROPHILS AND MONONUCLEAR CELLS

7.1 Eosinophils and neutrophils

Blood either from patients with eosinophilia or from healthy controls was drawn into plastic Universals containing 10 units of preservative free heparin (Evans Medical, Liverpool) per ml. The red cells were sedimented with 70% dextran (Lomodex 70, Fisons Pharmaceuticals, Loughborough); one part dextran to four parts blood v/v, for 60 min at room temperature. The leucocyte-rich plasma was then layered on to sodium metrizoate (Nyegaard & Co., As, Oslo, Norway), specific gravity 1.148 at 4°C, in 15 ml plastic conical centrifuge tubes and centrifuged at 4°C for 40 min at 400 x G (Day, 1970).

The pellet containing red blood cells and granulocytes was resuspended in ammonium chloride solution to lyse the erythrocytes (see below). The granulocytes were then washed twice in medium 199 (Flow Laboratories, Irvine, Ayrshire), pH 7.4, and the cell count was finally adjusted to 2 x 10^6/ml (for rosette experiments) in the same medium. These cell suspensions contained both eosinophils and neutrophils but were virtually free of mononuclear cells.

For the studies described in Fig. 29 in which suspensions of highly purified granulocytes of varying eosinophil/ neutrophil ratios were required, the technique of Wong and Wilson (1975) was used either alone or in combination with the method mentioned earlier. Wong and Wilson's method was modified slightly and was briefly as follows: two Ficoll-

Conray solutions A and B were prepared. Solution A contained 11.2 g of Ficoll (Pharmacia, Uppsala, Sweden) and 20 ml of Conray 280 (May & Baker, Dagenham) in 100 ml of distilled water; solution B contained 7.2 g of Ficoll and 20 ml of Conray 280 in 100 ml distilled water. These stock solutions were kept at 4°C and used for up to 7 days. On the day of the experiment 200 ml of blood were withdrawn from a normal volunteer and taken into plastic Universals containing 10 units/ml of preservative-free heparin. Ten millilitre portions of blood were layered on to 10 ml of solution B in plastic Universal containers and the red cells were allowed to sediment for 1 hr at room temperature. The leucocyte-rich plasma was removed and further layered, in 15 ml plastic centrifuge tubes, on to a gradient consisting of 3 ml of solution B which in turn had been gently placed on 3 ml of solution A so that an interface was formed between them. The tubes were then centrifuged at 1500 x G for 15 min at 4°C. Mononuclear cells sedimented between the plasma and solution B, and neutrophils at the interface between solution A and solution B, whilst the red cells and the eosinophils were pelleted at the bottom. The eosinophil and neutrophil layers were collected and the contaminating red cells were lysed using ammonium chloride (see below). Each cell suspension was washed twice in medium 199 and a differential white cell count was performed. To obtain granulocyte preparations of varying eosinophil enrichment, equal volumes of cell suspensions from different tubes, prepared either by this method or in combination with that of Day, were adjusted to 4 x 10^6 total granulocytes per ml

and mixed to obtain the required eosinophil/neutrophil ratio.

7.2 Preparation of mononuclear cells

These were prepared essentially by a modification of the method of Böyum (1968). Blood was collected in heparinized containers, diluted in 0.15 M NaCl (1:4) and then layered gently over Ficoll-Hypaque solution (specific gravity 1.077) in 15 ml conical plastic centrifuge tubes. The tubes were centrifuged for 25 min at 400 x G at 4°C. The mononuclear layer which sedimented at the interface was collected, washed twice in medium 199 and the cell count was adjusted to 2 x 10^6/ml (for rosette experiments) or 4 x 10^6 cells/ml (for enhancement and schistosomula experiments).

7.3 Lysis of contaminating red blood cells

Contaminating red cells were lysed by treatment with a hypotonic solution containing -

0.155 M NH_4Cl

0.01 M $KHCO_3$

0.0001 M EDTA

and its pH was adjusted to 7.4. Cell pellets were re-suspended in about 5 ml of ice cold lysis solution for 5 min at 0°C with frequent mixing. The mixture was then centrifuged for 10 min at 4°C. The leucocytes, which are now free of contaminating red cells, were washed twice in medium 199 and adjusted to the required concentration. The viability of these cells was always greater than 95%.

8.0 PREPARATION OF HUMAN RED CELLS

Venous blood samples were obtained from normal donors or laboratory personnel of known ABO and rhesus groups. The rhesus types were confirmed by routine blood banking methods. The following two types were used throughout in experiments involving the use of human red cells:

Group O rhesus positive OR_1R_1 (CDe/CDe)

OR_2R_2 (cDE/cDE)

8.1 Papainization of human red cells

Five drops of red cell suspension were mixed with an equal volume of 1% papain in sterile saline (BDH papain, papaya) for 4 min at room temperature. The cells were then washed four times in medium 199, counted, and adjusted as required.

9.0 EA AND EAC ROSETTE FORMATION

One-tenth millilitre of E, EA or EAC containing 1×10^8 cells/ml in $DGVB^{++}$ was added to an equal volume of granulocyte or mononuclear cell suspension in medium 199 containing 2×10^6 cells/ml. The mixture was centrifuged at 100 x G for 10 min at 4^oC. These mixtures, with the undisturbed pellets, were then incubated at 4^oC (for the E rosettes), 0^oC (for EA rosettes) or 37^oC (for EAC rosettes) for the optimum incubation time (see below).

9.1 Rosette inhibition by human heat-aggregated IgG

The granulocytes used in these experiments were adjusted to a concentration of 4×10^6/ml in medium 199. Equal volumes (0.5 ml) of granulocyte suspension and aggregated IgG preparation (containing 1.2 mg protein/ml) were mixed. The mixture was incubated for 30 min at 4^oC with frequent shaking. The cells were then washed three times in medium 199 and their concentration was adjusted to 2×10^6/ml before they were used in the rosette assay.

9.2 Preparation and counting of rosette slides

The pellets, containing both leucocytes and indicator red cells, prepared as described above, were gently resuspended and smears were prepared in duplicate on clean glass slides. These were dried quickly in air (using a fan or a hair-dryer), fixed in 95% methanol and stained with May Grunwald/Giemsa. The staining procedure was as follows:

(i) Slides were placed in 50% May-Grunwald solution for 3 min.

(ii) They were transferred, without rinsing, to 10% Giemsa solution for 3 min.

(iii) They were destained in buffered distilled water (pH 6.8) for 2 min.

During staining the slides were usually examined for the staining density and might be destained for a longer period if required. They were then allowed to dry and either sprayed with liquid coverglass (Trycolac) or covered by D.P.X. mounting medium and sealed with coverslips.

Only leucocytes with three or more adherent red cells were termed rosettes. In each slide 200 leucocytes were counted and the number of rosetting cells was expressed as a percentage of the total number of cells counted.

10.0 IMMUNOFLUORESCENCE STUDIES

10.1 Detection of receptors for heat-aggregated human IgG on human granulocytes

For these experiments the granulocyte suspensions containing 5 x 10^6/ml in medium 199 were used. Before use the cells were washed once in 'FA buffer' (Difco Laboratories, Detroit, Michigan, U.S.A.) and 0.5 ml of the granulocyte suspension was mixed with an equal volume of heat-aggregated human IgG, native IgG or as a control, heat-aggregated bovine serum albumin, all containing 1.2 mg/ml protein. The mixtures were incubated at 0°C for 30 min. The cells were then washed three times in FA buffer at 4°C. Fluorescent labelled rabbit anti-human IgG (Miles Laboratories, Slough) was added to the cell suspensions and the mixtures were incubated for a further 30 min at 0°C. The cells were again washed three times in cold FA buffer and then re-suspended in two drops of FA mounting medium (buffered glycerol) (Difco Laboratories). One drop of the cell suspension was mounted on a clean glass slide under a coverslip and the edges were sealed with nail varnish. The slides (prepared in duplicate for each sample) were then viewed under ultraviolet light and the number of fluorescing cells were counted. Only cells with two or more peripheral fluorescing sites (i.e. non-granular fluorescence) were deemed positive. In each slide 200 cells were counted and the granulocytes showing positive fluorescence were expressed as a percentage of the total number of the granulocytes counted.

10.2 Detection of various complement components bound to the tegument of schistosomula of Schistosoma mansoni

One millilitre of schistosomula suspension (containing 1000 organisms) was incubated at 37°C for 1 hr with an equal volume of either fresh normal human serum, human C2-deficient serum, heat inactivated human serum or the appropriate control (Table VIII). After washing twice in phosphate buffered saline (PBS) the schistosomula were incubated at 4°C with fluorescent anti-human C4 or anti-human C3 for 30 min. Finally the schistosomula were washed three times in PBS, mounted on clean glass slides and examined by ultraviolet light.

11.0 TREATMENT OF LEUCOCYTES WITH VARIOUS PHARMACOLOGICAL AGENTS

The leucocyte cell concentration was adjusted to 4×10^6 cell/ml in medium 199, pH 7.4. Equal volumes of the leucocyte suspensions and solutions containing various concentrations of the pharmacological agents under study, or control medium alone, were mixed and incubated in a shaking water bath at 37°C for 1 hr. The cells were then washed twice in medium 199 and the concentrations were adjusted finally to 2×10^6/ml in the same medium. A portion (0.1 ml) of EAG or EAC red cells containing 1×10^8 red cells/ml was then added to an equal volume of the leucocyte cell suspension. The mixtures were centrifuged at 100 x G for 10 min at 4°C and the pellets were incubated either at 0°C (for EA rosettes) or 37°C (for EAC rosettes) for 30 min. The pellets were gently resuspended and smears were prepared on clean glass slides in duplicate. The slides were dried quickly in air, fixed in methanol and stained with May Grunwald/Giemsa. Leucocytes with three or more adherent sheep red cells were counted as rosettes. In each slide 200 cells were counted and the number of rosetting cells was expressed as a percentage of the total number of leucocytes counted.

12.0 HUMAN LUNG ANAPHYLACTIC DIFFUSATE

This was kindly prepared by Mr. J. Stewart, Department of Pathology, University of Edinburgh, from human lung obtained at surgery, usually in association with bronchogenic carcinoma, according to the method described by Turnbull et al (1976). Briefly, lung fragments were washed in Tyrode's buffer and each gram wet weight of sliced lung fragment was incubated with 5.4 ml of a 1 in 8 dilution of serum from an individual sensitive to Timothy grass pollen. After an incubation period of 18 hr at room temperature the lung fragments were washed twice in Tyrode's buffer and challenged with 9 ml of the same buffer, containing 0.2 μg/ml of purified Timothy grass pollen extract (TGP). Following incubation for 15 min at 37°C the diffusate was removed and stored at -85°C until use. Appropriate controls in which unsensitized lung was challenged with the same amount of antigen (TGP) or sensitized lung was challenged with Tyrode's buffer alone, were prepared in parallel. The histamine content in these diffusates was assayed on atropinized guinea pig ileum according to the method of Brocklehurst (1960).

13.0 PREPARATION OF SCHISTOSOMULA

13.1 The parasite cycle

A Puerto Rican strain of Schistosoma mansoni was maintained in laboratory-bred Biomphalaria glabarata and outbred Parkes mice according to the method of Smithers and Terry (1965).

13.2 Mechanical transformation of cercariae to schistosomula

Schistosomula were kindly prepared by Dr. S.R. Smithers, Division of Parasitology, National Institute for Medical Research, Mill Hill, London, according to the method of Ramalho-Pinto et al (1974). Briefly, cercariae freshly shed from snails were concentrated by the addition of penicillin-streptomycin, followed by chilling and centrifuging at 1000 rpm for 30 sec. One millilitre of deionized water was added to the pellet and the suspension thoroughly mixed using a Vortex mixer (Fisons Scientific, Loughborough) for 1 min. This effected the rupture of tails from bodies, which were subsequently separated by sedimentation in Hank's buffered salt solution. The cercarial bodies were then incubated for 3 hr in RPMI-1640, 20 mM Hepes. The schistosomula were transported by train from London to Edinburgh at ambient temperature, and were used in the experiments described here approximately 24 hr after their preparation.

13.3 Preparation of complement coated schistosomula

One thousand schistosomula (Sch) contained in 1 ml were sensitized by incubation with 1 ml of human anti-

schistosomula serum (A) for 3 hr at 37°C and washed twice in $DGVB^{++}$, pH 7.4 (SchA). SchA were then incubated for 15 min with 40,000 effective molecules per schistosomulum of human C1 at 37°C and washed once in $DGVB^{++}$(SchAC1). For the preparation of SchAC14, 40,000 molecules of purified human C4 were added to SchAC1 and the mixture was incubated for 30 min at 37°C and washed twice in $DGVB^{++}$. Five thousand effective molecules of human C2 were then added per SchAC14 and the mixture was incubated at 30°C for 10 min without washing (SchAC142). Increasing concentrations of purified human C3 (i.e. 1×10^5, 2×10^5, 3×10^5 and 4×10^5 effective molecules per schistosomulum) were added to SchAC142 and the mixtures were incubated for 30 min at 37°C and washed twice (SchAC1423). Bound C3b was identified on schistosomula by the use of fluorescent anti-human C3.

14.0 SCHISTOSOMULA KILLING ASSAY

The experiments were performed in flat-bottomed microtitre plates (Sterilin, Middlesex). Except in experiments performed to determine the optimal effector cell:target ratios, the ratio of leucocytes to schistosomula was 4000:1. The reaction volume was 0.4 ml for each experimental system and each treatment was performed in duplicate and sometimes in quadruplicate. These experimental systems were (a) 'antibody alone', 0.1 ml of baboon, human anti-schistosomula serum or its IgG fraction (in medium 199), 0.1 ml of schistosomula, 0.1 ml of leucocytes and 0.1 ml of medium 199; (b) 'complement alone', 0.1 ml of fresh autologous (in respect to the leucocyte donor) serum, 0.1 ml of schistosomula, 0.1 ml of leucocytes and 0.1 ml of medium 199; (c) 'antibody and complement', 0.1 ml of antibody (as in (a)), 0.1 ml of complement (as in (b)), 0.1 ml of schistosomula and 0.1 ml of leucocytes. The controls were schistosomula (0.1 ml) incubated with (i) medium 199 (0.3 ml), (ii) leucocytes (granulocytes or mononuclear cells) (0.1 ml) and medium 199 (0.2 ml) or (iii) leucocytes (0.1 ml), heat inactivated (56°C for 1 hr) fresh autologous serum (0.1 ml) and medium 199 (Q2 ml). In addition, schistosomula (0.1 ml) were also incubated with antibody as in (a), fresh autologous serum (0.1 ml) and medium 199 (0.1 ml) in the absence of leucocytes.

The microtitre plates were incubated for 18 hr at 37°C in an atmosphere of 5% CO_2 and 95% air. Schistosomulum death was determined by direct light microscopy. Only motile schistosomula (Sch^m) or schistosomula killed by

adherent leucocytes (Sch^d) were counted. Dead schistosomula were recognised by their absence of movement, although in some experiments cytotoxicity was confirmed by their inability to exclude trypan blue. Killing was expressed as a percentage:

$$\frac{Sch^d}{Sch^d + Sch^m} \times 100$$

Some non-viable schistosomula (Sch^{nv}) were invariably present in the suspension prior to treatment and their number usually increased slightly during the experimental period. However, they were easily recognised by their relative opacity, flattened appearance and lack of motility. The Sch^{nv} present in those control or test wells containing leucocytes were not counted. The controls containing antibody and complement in the absence of leucocytes, were calculated by subtracting the number of Sch^{nv} contained in medium 199 alone.

CHAPTER IV - RESULTS

SECTION I - DETECTION OF RECEPTORS FOR IMMUNOGLOBULINS ON HUMAN EOSINOPHILS AND NEUTROPHILS

SECTION I - CONTENTS

1.0 INTRODUCTION

The presence of membrane surface receptors for specific immunoglobulins and various complement components has been identified on a variety of human and animal haemopoietic cells. These receptors appear to be part of a cell membrane recognition system which may be very important. In the case of the lymphocyte, the receptors may represent the recognition link between an antigen on the outside of the cell and the genetic capacity of the lymphocyte to respond to stimulation. For phagocytic cells the receptors may be required for membrane attachment and subsequent phagocytosis. In addition, these surface receptors have permitted more precise identification of subpopulations of cells, e.g. T and B lymphocytes.

Most of the studies on surface receptors relate to cells of the lymphoid series and mononuclear phagocytes, although the presence of surface receptors for IgG and complement on human or animal granulocytes was also described. For example, the presence of receptors for human IgG (Messner and Jelinek, 1970), C3b (Eden et al, 1973) and C4 (Ross and Polley, 1974) on human neutrophils has been described. Similarly, the presence of IgG (Tai and Spry, 1976) and C3 receptors on human eosinophils has been reported (Tai and Spry, 1976; Sher and Glover, 1976).

The present studies, undertaken to define more precisely the presence of immunoglobulin and complement receptors on human eosinophils, were mainly stimulated by the report of Butterworth et al (1975) who showed that human eosinophils from normal individuals were the main

effector cells in antibody-dependent killing of schistosomula *in vitro*.

In the first two sections of this Chapter the results of experiments performed to study the presence of receptors for immunoglobulins and various human complement components on human eosinophils and neutrophils are described. The main experimental procedure used in these studies was the rosette technique although in some experiments the immunofluorescent technique was used.

2.0 DETECTION OF RECEPTORS FOR IgG ON HUMAN EOSINOPHILS AND NEUTROPHILS

2.1 Determination of optimal experimental conditions: Time course and temperature

Early experiments were designed to establish the optimal experimental conditions required for maximum rosette formation between human eosinophils and neutrophils and sheep red blood cells sensitized with rabbit IgG (EA_G^{rab}). Rosette formation by both eosinophils and neutrophils with EA_G^{rab} was found to be temperature and time dependent (Figs. 1 and 2). With an increase in the incubation time there was a corresponding increase in rosette formation which was maximal at 30 min (81% for neutrophils and 28% for eosinophils) after which the percentage of rosettes for both cell types remained relatively constant. In some experiments (not shown) even when the incubation time was increased for up to 90 min, rosette formation by both eosinophils and neutrophils was not significantly affected.

Rosette formation by these two cell types with EA_G^{rab} was tested at three different temperature, 0^oC, room temperature, and at 37^oC. It was found that EA_G^{rab} rosette formation was maximal for both eosinophils and neutrophils at 0^oC (Figs. 1 and 2). At higher temperatures, i.e. at room temperature and 37^oC, rosette formation was markedly decreased and most of the neutrophils and eosinophils showed evidence of having ingested sensitized red blood cells.

At the optimum experimental conditions, i.e. 0^oC and after 30 min incubation, the percentage of rosetting neutrophils was approximately two and one-half times that for eosinophils.

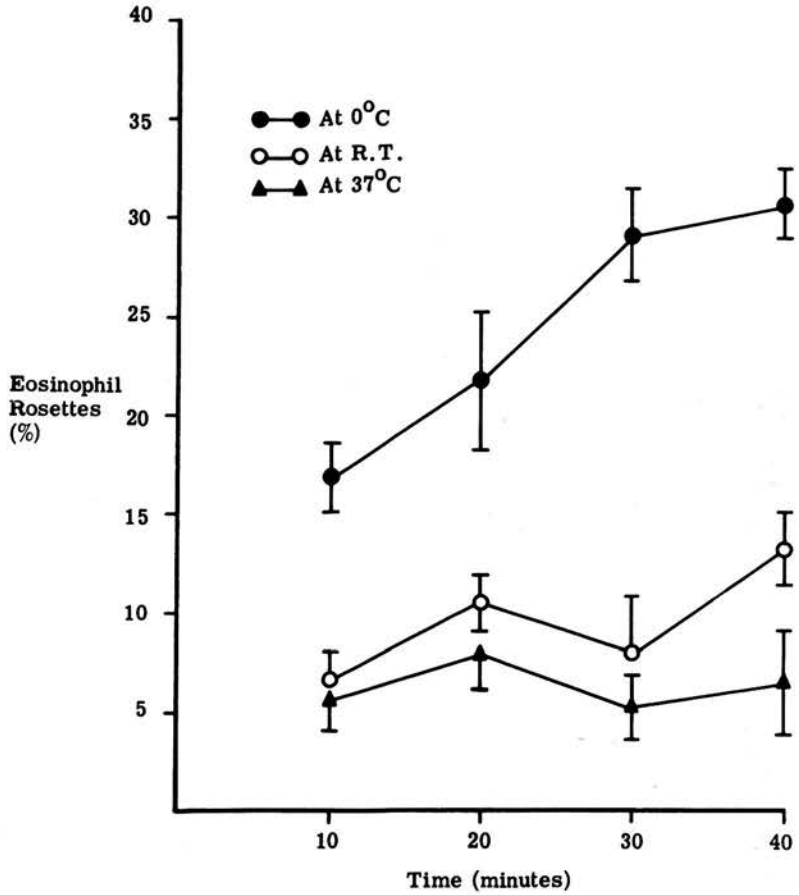

Fig. 1

The effect of variation of temperature and time of incubation on rosette formation by human eosinophils with EA_G^{rab}.

The points represent the mean (± 1 S.D.) of five experiments.

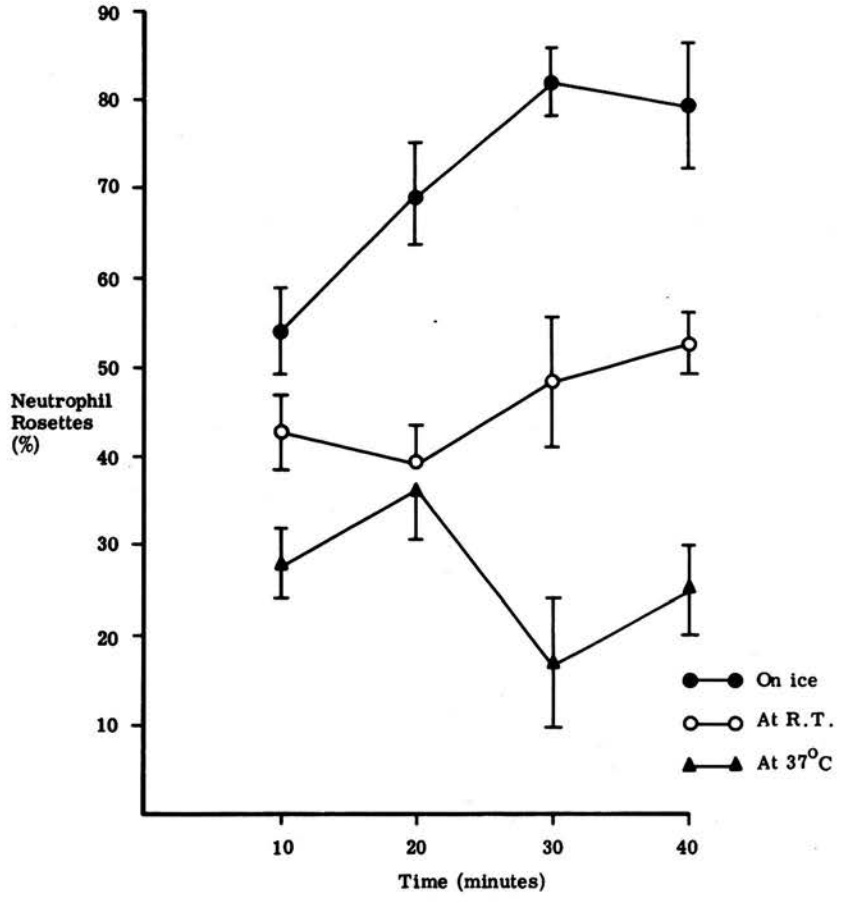

Fig. 2

The effect of variation of temperature and time of incubation on rosette formation by human neutrophils with EA_G^{rab}.

The points represent the mean (± 1 S.D.) of five experiments.

It was decided to use 0°C and 30 min as optimal experimental conditions to detect eosinophil and neutrophil EA_G^{rab} rosette formation in the subsequent experiments.

2.2 Effect of increasing IgG concentrations on eosinophil and neutrophil rosette formation with EA_G^{rab}

In these experiments, sheep red cells were sensitized with increasing amounts of IgG prior to incubation with eosinophils and neutrophils. The percentages of rosette formation by both eosinophils and neutrophils was directly dependent on the amount of IgG (Fig. 3). Again it was found that using the maximum subagglutination IgG titre and at the optimal conditions, the percentage of rosetting neutrophils was between two and three times greater than the percentage of eosinophil rosettes.

2.3 Inhibition of EA_G^{rab} rosette formation by eosinophils and neutrophils by human heat aggregated IgG

When human neutrophils and eosinophils were pre-incubated at 0°C with a constant amount of heat aggregated IgG (as described in the methods) and then tested for EA_G^{rab} rosette formation by incubating them at 0°C with sheep red cells sensitized by increasing concentrations of rabbit IgG, there was a significant decrease in the percentage of rosettes detected with both cell types (Fig. 4).

2.4 Detection of receptors for human IgG by immunofluorescence

The presence of membrane surface receptors for human IgG was tested directly by treating the granulocyte cell suspension with heat aggregated human IgG and then counting the number of

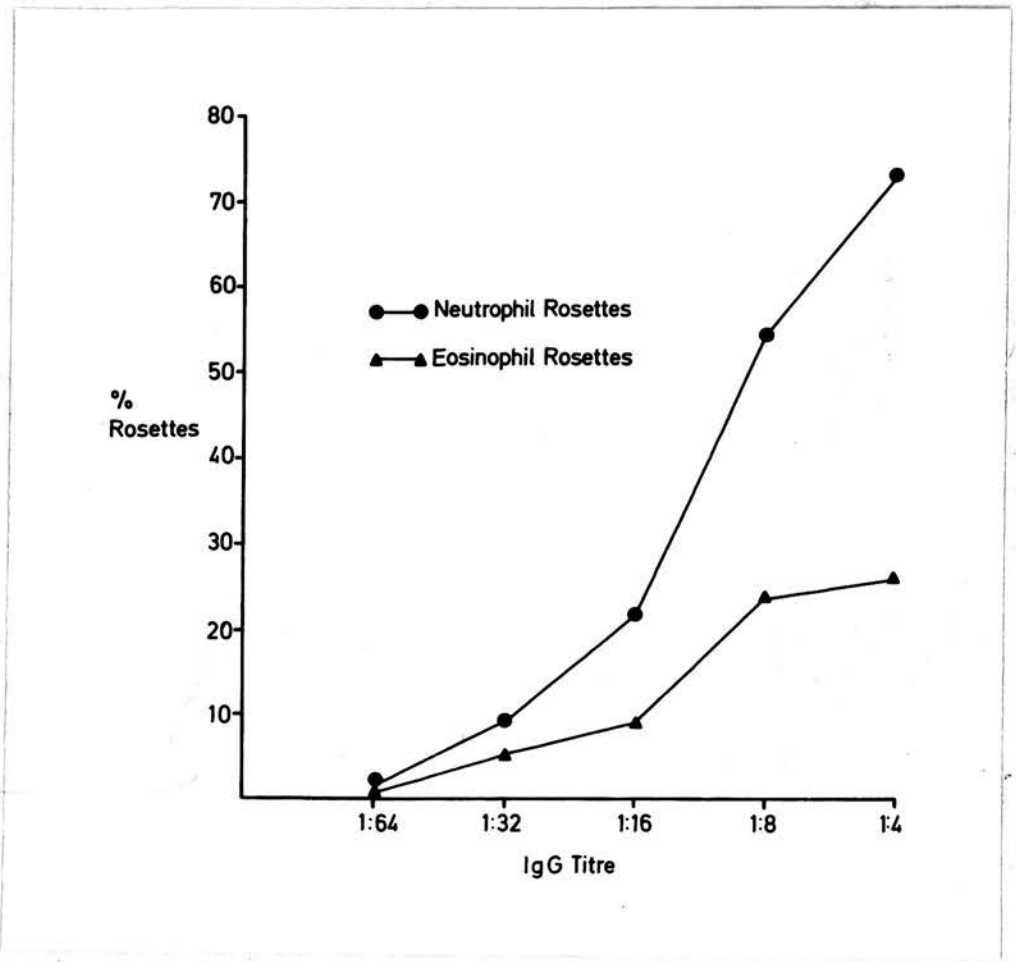

Fig. 3

The effect of increasing concentrations of rabbit IgG on human neutrophil and eosinophil rosette formation with EA_G^{rab}.

The points represent the mean of two experiments.

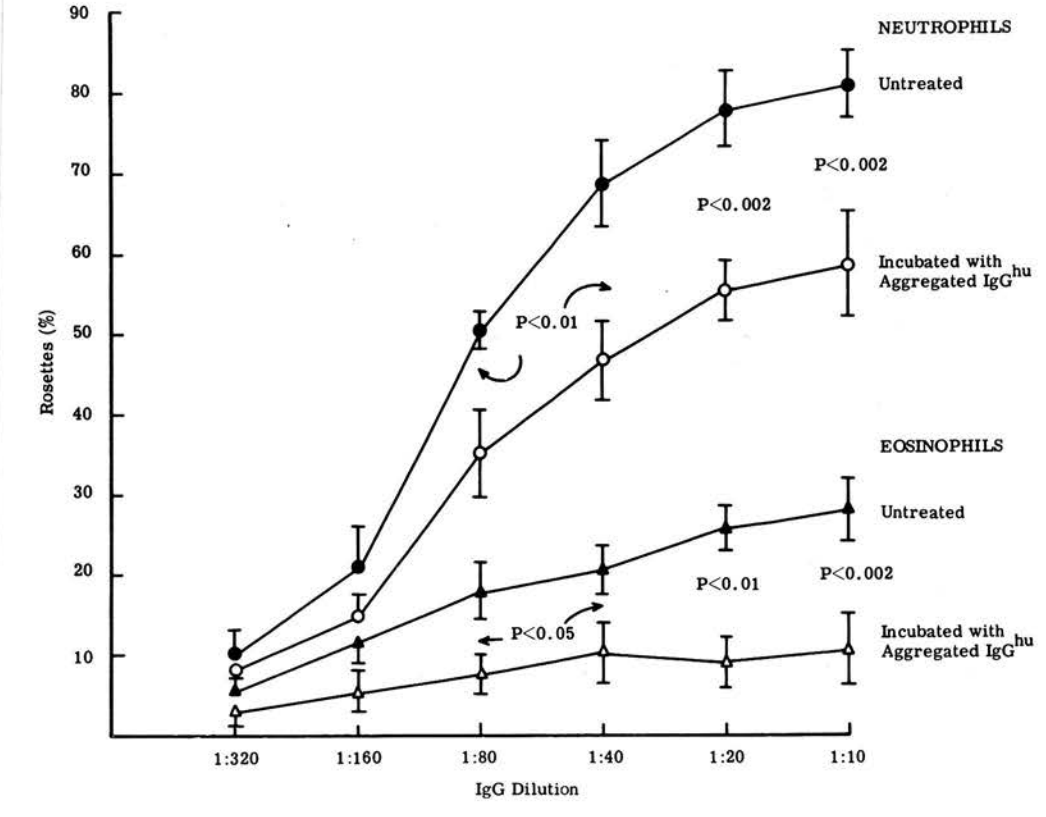

Fig. 4

The effect of increasing concentrations of rabbit IgG on human neutrophil and eosinophil rosette formation with EA^{rab}_{G} and their inhibition by heat-aggregated human IgG.

The points represent the mean (± 1 S.D.) of five experiments. The degree of significance of inhibition with heat-aggregated human IgG is expressed as p value as calculated by the Student t-test.

Plate I

Eosinophil rosette formation with EA_G^{rab}

(May-Grunwald/Giemsa, x 1000 magnification)

cells showing positive peripheral fluorescence after the addition of fluorescein-labelled monospecific rabbit anti-human IgG (Fig. 5). With both cell types the number of fluorescing cells with heat aggregated IgG was significantly greater ($p < 0.001$) than with native unaggregated human IgG or with heat aggregated bovine serum albumin. As with EA_G^{rab} rosettes, the percentage of fluorescing neutrophils was about two and one-half times greater than that of the fluorescing eosinophils.

2.5 Detection of receptors for human IgG using human Rhesus positive red cells sensitized with anti-D sera

In these experiments human red cells (O R_1R_1 or O R_2R_2) were sensitized with human anti-D sera and then incubated with eosinophils, neutrophils and monocytes to detect rosette formation.

There was no rosette formation between these red cells sensitized with any of the anti-D sera tested and either eosinophils or neutrophils. However, in all the experiments performed the monocytes formed rosettes. The mean percentage of rosettes formed by monocytes was 47.5% ± 6.5 compared to less than 5% with either the eosinophils or neutrophils (Fig. 6).

In a second series of experiments human red cells were treated by the enzyme, papain, before sensitization. The use of such enzymes is thought to enhance antibody-red cell interactions, especially with antibody of the Rhesus blood group. However, again there was no rosette formation between the papainized red cells and eosinophils or neutrophils. Monocytes tested at the same time using the

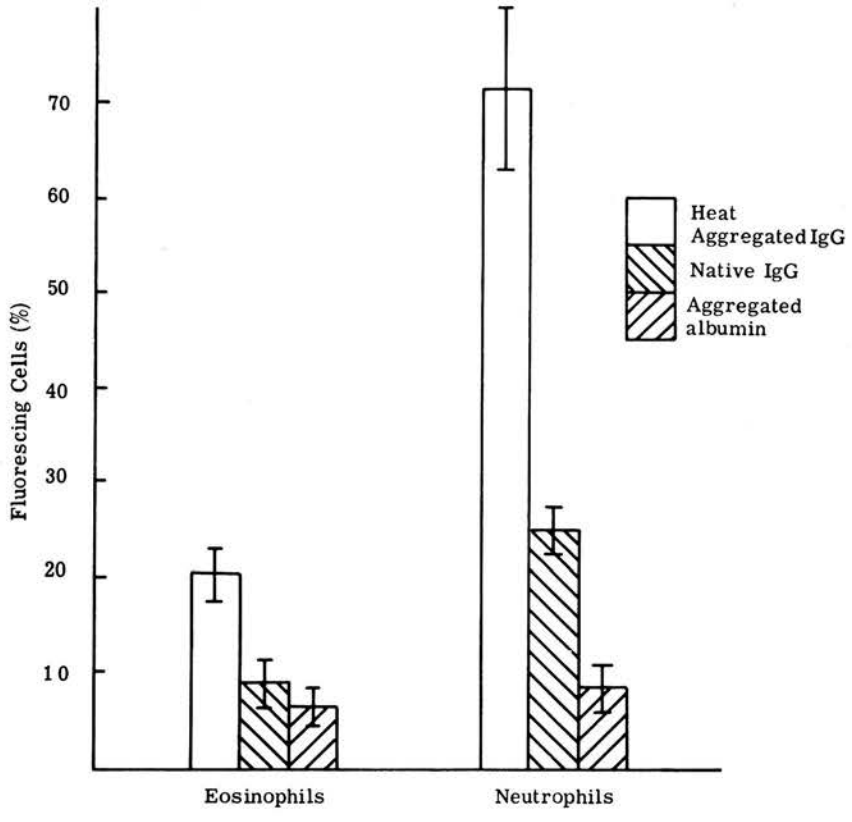

Fig. 5

The percentage of fluorescing eosinophils and neutrophils after treatment with heat-aggregated IgG, or controls, and fluorescent anti-human IgG.

The bars represent the mean (± 1 S.D.) of seven experiments.

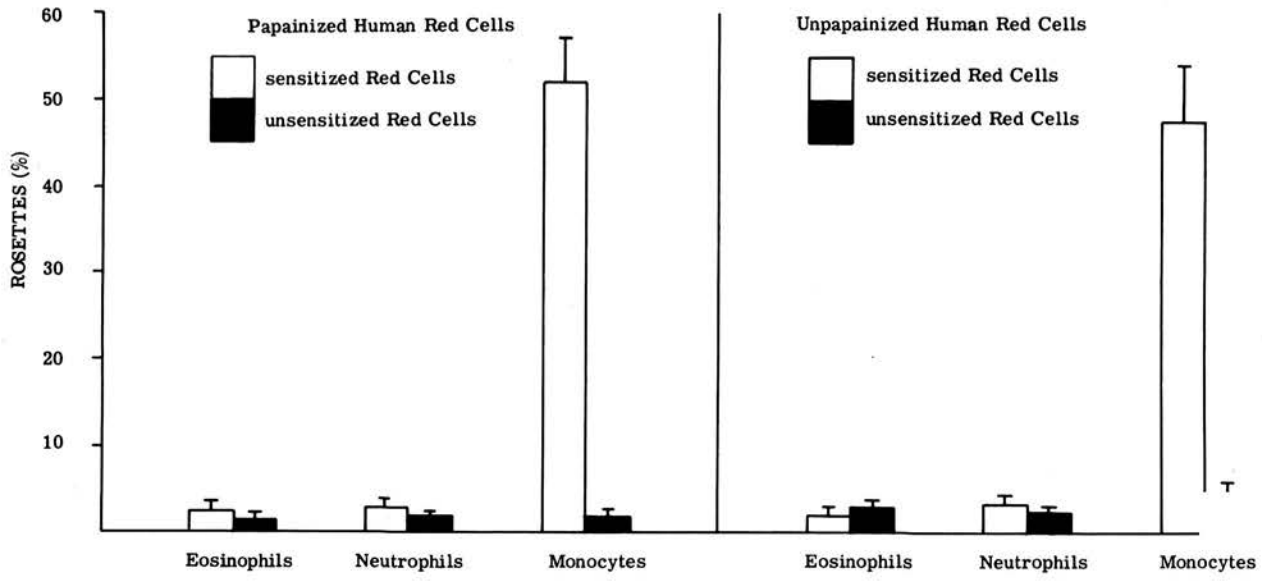

Fig. 6

The effect of papainization of human red cells (group O Rh +ve) on rosette formation by human eosinophils, neutrophils and monocytes with sensitized or unsensitized human red cells.

The bars represent the mean (± 1 S.D.) of four experiments.

same papainized red cells formed similar numbers of rosettes to those detectable using unpapainized erythrocytes (52% ± 5.0) (Fig. 6).

2.6 Receptors for IgG on eosinophils from healthy donors and patients with eosinophilia

Eosinophils obtained from healthy blood donors and eosinophils from patients with eosinophilia of various aetiology were tested for their capacity to form rosettes with EA_G^{rab}. Before donors were considered healthy for these experiments they had to fulfil the following criteria:

(a) they should be clinically asymptomatic at the time of the experiment;

(b) there should be no previous history of conditions associated with blood eosinophilia;

(c) the blood eosinophil count should be less than 400 cells per cu.mm on the day of the experiment.

In these experiments 15 healthy volunteers fulfilling the above criteria were compared with 15 patients with eosinophilia associated with various causes, the majority being in relation to an exogenous antigen.

Eosinophils from both healthy donors and from eosinophilic patients formed rosettes with EA_G^{rab}. There was no significant difference in the percentage of rosetting eosinophils between the two groups (Fig. 7). When the neutrophils from the two groups were compared as regards to their capacity to form EA_G^{rab} rosettes, there was also no significant difference in the percentage of neutrophil rosettes.

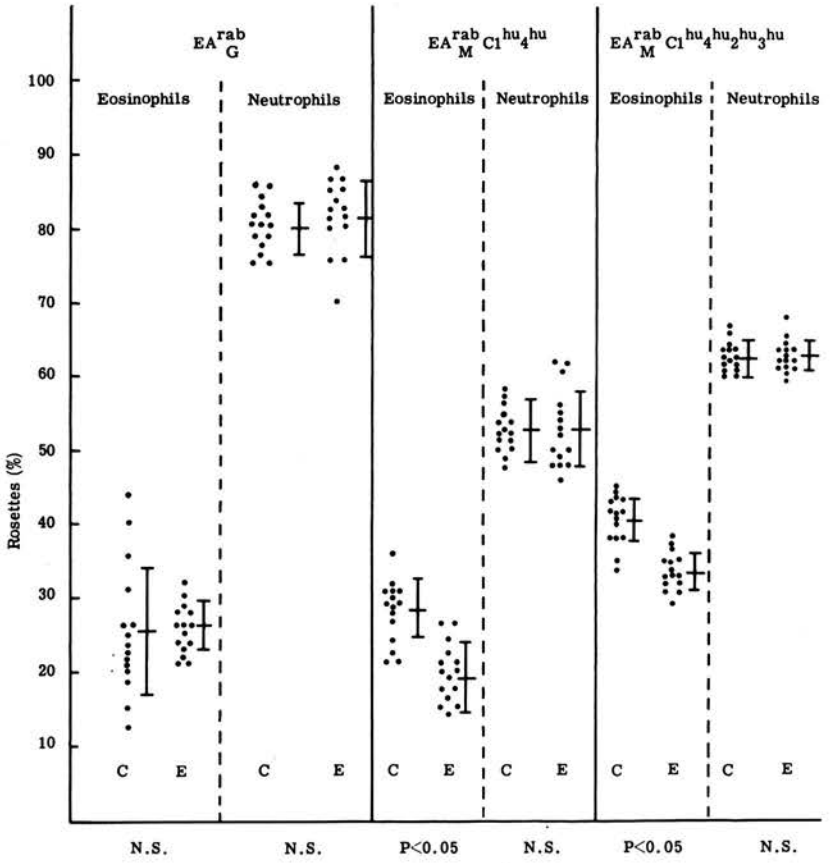

Fig. 7

The percentage of eosinophil or neutrophil rosettes with EA^{rab}_{G}, EAC14 or EAC3b as compared between healthy normal controls (C) and patients with eosinophilia (E). The EAC14 cells were prepared with 4000 effective molecules of C4 and the EAC3b cells were prepared with 400 effective molecules of C4 per cell.

The bars represent the mean (± 1 S.D.). The p values were calculated by the Student t-test.

N.S. = not significant.

The percentage of either blood eosinophilia or the percentage of eosinophil and neutrophil EA_G^{rab} rosettes in 15 patients was unrelated to the disease states. In addition, there was no association between the percentage of eosinophilia and the percentage of EA_G^{rab} neutrophil or eosinophil rosettes (Table IV).

3.0 DETECTION OF RECEPTORS FOR IgM AND SHEEP RED BLOOD CELLS ON HUMAN EOSINOPHILS AND NEUTROPHILS

3.1 Eosinophil and neutrophil EA_M^{rab} rosettes

Sheep red cells sensitized with rabbit IgM were tested for their capacity to form rosettes with human eosinophils and neutrophils. The experiments were performed at both 37°C and at 0°C. The ability of eosinophils to form rosettes with both EA_M^{rab} and EA_G^{rab} is shown in Fig. 8. Using either temperatures there was no rosette formation by eosinophils and EA_M^{rab}. In contrast, eosinophils formed rosettes with EA_G^{rab} at both temperatures, although rosette formation at 0°C was significantly greater. Similarly, in all experiments neutrophils formed rosettes with EA_G^{rab} but not with EA_M^{rab}.

It was concluded that, under the experimental conditions used, there were no receptors for rabbit IgM on human eosinophils or neutrophils and, therefore, red cells sensitized with rabbit IgM were used for the preparation of complement coated intermediates so that when testing for rosette formation with various complement components, there was no possibility that the rosettes which formed were due to the presence of IgM receptors.

3.2 Eosinophil and neutrophil rosettes with untreated sheep red cells

Unsensitized sheep red cells were tested for their capacity to form rosettes with eosinophils, neutrophils and lymphocytes under the experimental conditions described. In all the experiments performed lymphocytes formed rosettes

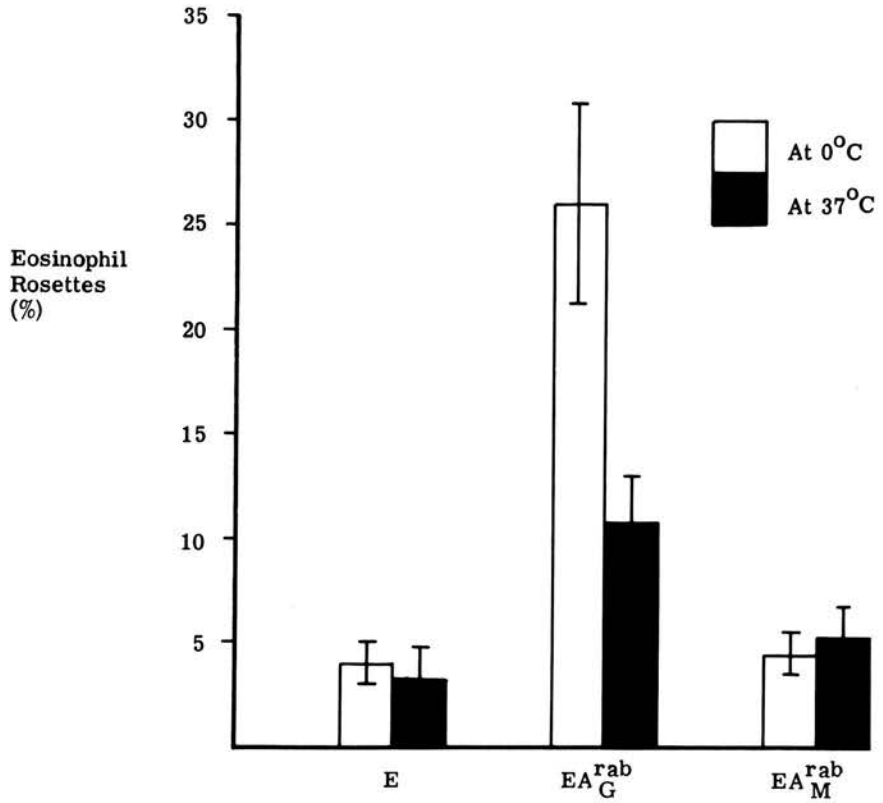

Fig. 8

Rosette formation by human eosinophils with untreated sheep red cells (E), EA_G^{rab} and EA_M^{rab}.

The bars represent the mean (± 1 S.D.) of five experiments.

with untreated sheep red cells whilst neither eosinophils nor neutrophils showed any significant rosette formation (Fig. 9). The mean (± 1 S.D.) percentage rosettes was 46.0 ± 5.5 for lymphocytes, 6.0 ± 2.0 for neutrophils and 7.0 ± 2.5 for eosinophils.

3.3 Eosinophil and neutrophil rosettes with AET-treated sheep red cells

The rosettes formed between T lymphocytes and untreated sheep red blood cells are known to be fragile and can easily break. Therefore, several possible methods for stabilizing the red cell binding have been described. One of these methods involves the treatment of the sheep red cells with aminoethylisothiouronium bromide hydrobromide (AET) which has been reported to enhance markedly their T cell binding capacity (Kaplan and Clark, 1974).

When sheep red cells were treated with AET and then tested for E rosette formation with human eosinophils and neutrophils there was no difference between their ability to form rosettes before and after treatment (Fig. 9). The mean (± 1 S.D.) E (AET-treated) percentage rosettes was 8.0 ± 3.0 for neutrophils and 6.5 ± 3.0 for eosinophils. With lymphocytes after AET treatment the mean (± 1 S.D.) percentage E rosettes was 58.5 ± 5.0.

Therefore, although the AET treatment of sheep red blood cells markedly enhanced their lymphocyte binding, it had no effect on rosette formation by either the eosinophil or the neutrophil.

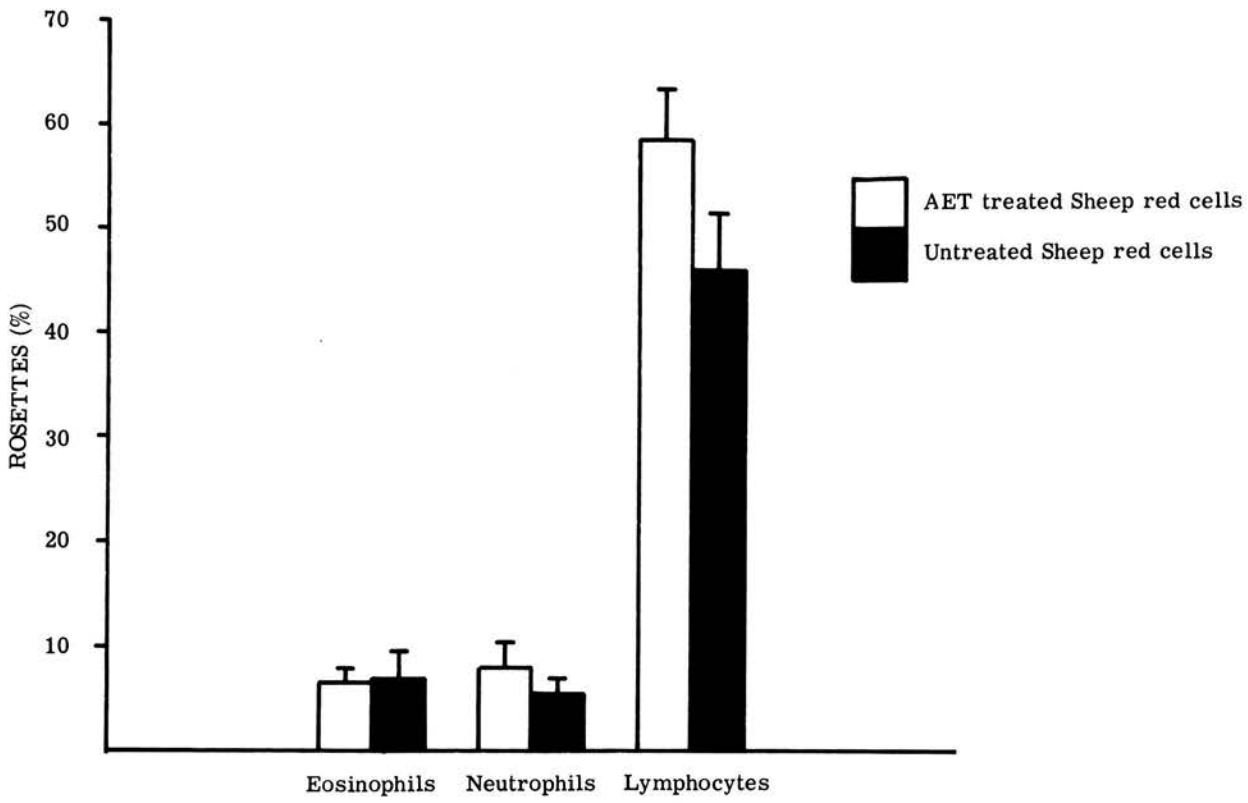

Fig. 9

Rosette formation by human eosinophils, neutrophils and lymphocytes with AET-treated sheep red cells and with untreated sheep red cells.

The bars represent the mean (± 1 S.D.) of seven experiments.

4.0 SUMMARY

Human eosinophils and neutrophils formed rosettes with EA_G^{rab}. Rosette formation was directly related to the amount of sensitizing antibody and was inhibited by heat aggregated human IgG.

Receptors for human IgG on human eosinophils and neutrophils were also demonstrated by using heat aggregated human IgG and fluorescent-labelled anti-human IgG.

Under the experimental conditions described in this study, no rosette formation could be demonstrated between human eosinophils and neutrophils with either (i) human red cells (O R_1R_1 or O R_2R_2) sensitized with various sources of anti-D, (ii) sheep red cells sensitized with rabbit IgM (EA_M^{rab}), (iii) untreated sheep red cells, or (iv) sheep red cells treated with AET.

There was no significant difference in the percentage of eosinophils or neutrophils forming rosettes with EA_G^{rab} when cells from patients with eosinophilia of various aetiology were compared to cells from healthy individuals.

SECTION II - DETECTION OF RECEPTORS FOR VARIOUS HUMAN COMPLEMENT COMPONENTS ON HUMAN EOSINOPHILS AND NEUTROPHILS

SECTION II - CONTENTS

1.0 DETERMINATION OF OPTIMAL EXPERIMENTAL CONDITIONS

1.1 Time course and temperature

Sheep red cells sensitized with rabbit IgM (EA_M^{rab}) were used in these experiments. EA_M^{rab} coated with purified human C1 (EAC1) did not form rosettes with either eosinophils or neutrophils at either 0°C or 37°C. When purified human C4 was added to EAC1 to form EAC14 intermediate, rosette formation with both eosinophils and neutrophils was observed. When C2 was added to EAC14 to form EAC142, no rosettes were detected. With the addition of C3 to EAC142 to form EAC1423, rosette formation was again detected with both eosinophils and neutrophils. From these preliminary experiments it was concluded that neutrophils and eosinophils can form rosettes only with EAC14 and EAC1423 cells. It was, therefore, decided that the optimal experimental conditions for EAC14 and EAC1423 rosette formation by eosinophils and neutrophils should be determined.

The indicator red cells, i.e. EAC14 and EAC1423, were tested for rosette formation by eosinophils and neutrophils at 0°C, room temperature and at 37°C at various incubation times. For both intermediates it was found that the rosettes were better expressed at 37°C than at room temperature or at 0°C (Figs. 10, 11, 12 and 13). In addition, it was found that there was a time-dependent increase in the rosette formation by both eosinophils and neutrophils with EAC14 and EAC1423 up to 30 min after which the percentage of rosettes remained relatively constant (Figs. 10, 11, 12 and 13). It was, therefore, decided to use an incubation time of 30 min

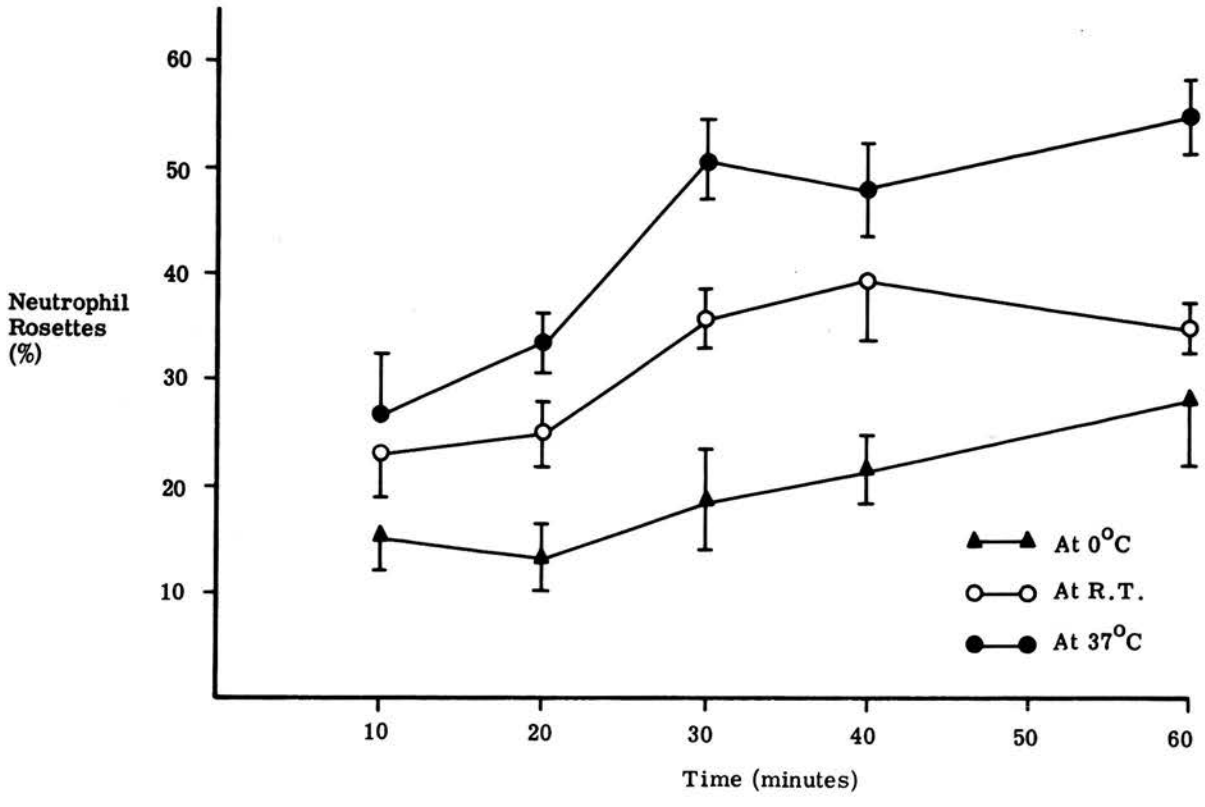

Fig. 10

The effect of variation of temperature and time of incubation on EAC14 rosette formation by human neutrophils.

The points represent the mean (± 1 S.D.) of five experiments.

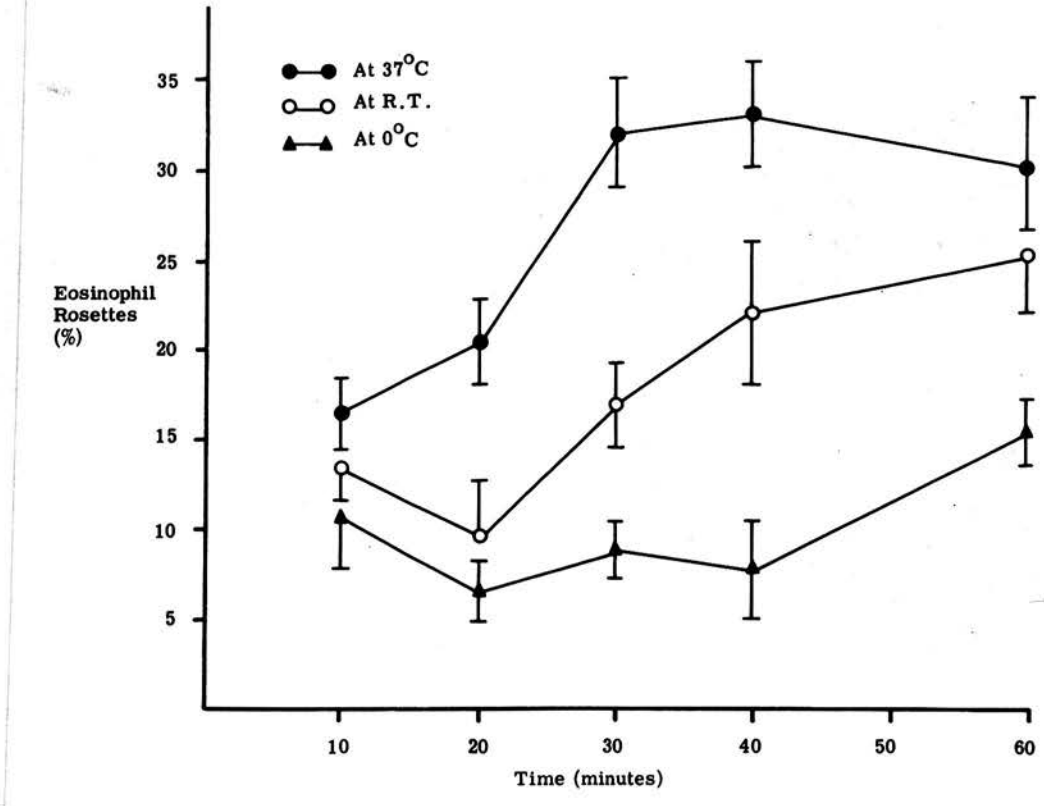

Fig. 11

The effect of variation of temperature and time of incubation on EAC14 rosette formation by human eosinophils.

The points represent the mean (± 1 S.D.) of five experiments.

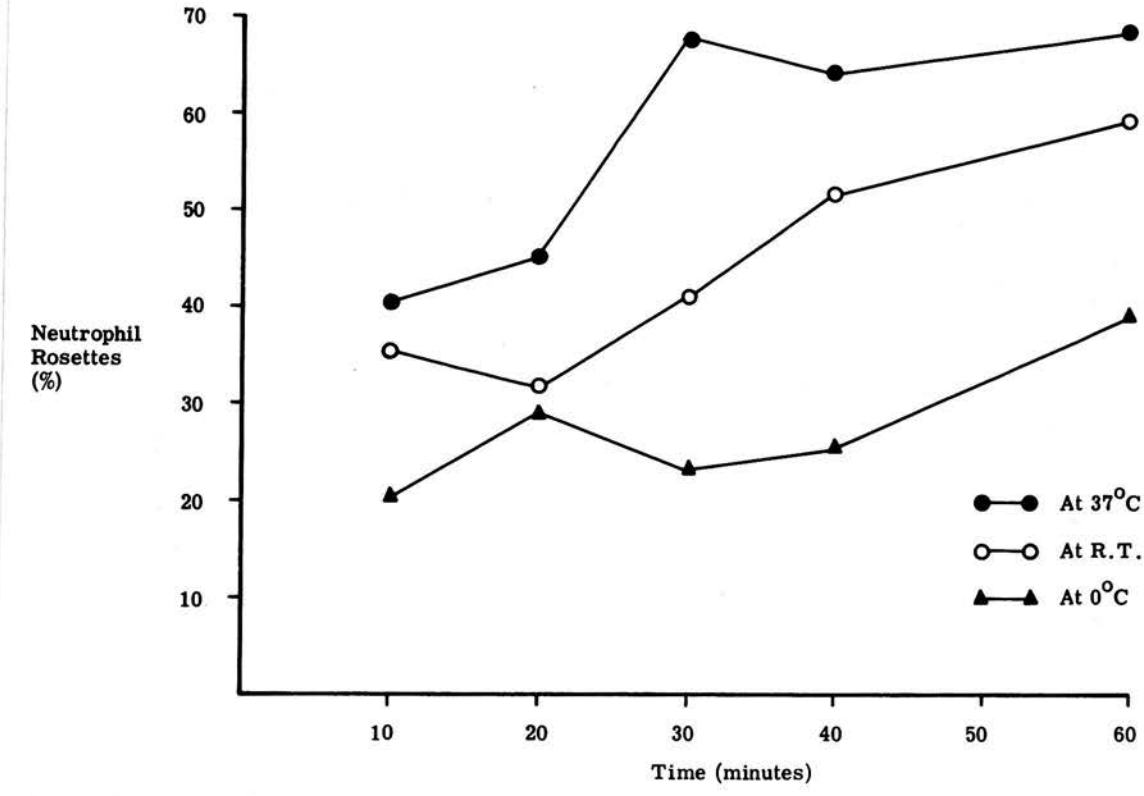

Fig. 12

The effect of variation of temperature and time of incubation on EAC3b rosette formation by human neutrophils.

The points represent the mean of two experiments.

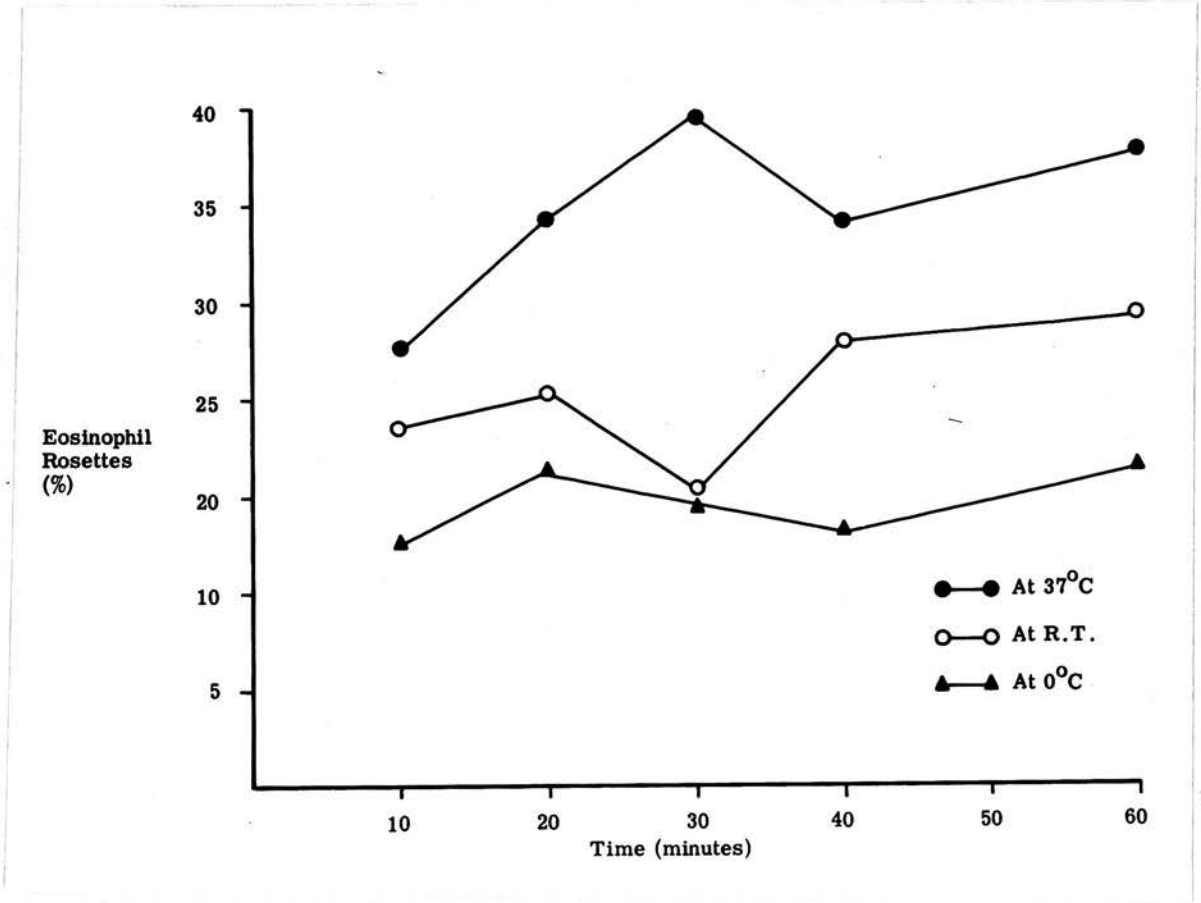

Fig. 13

The effect of variation of temperature and time of incubation on EAC3b rosette formation by human eosinophils.

The points represent the mean of two experiments.

at 37°C as optimal conditions for the detection of complement rosettes by eosinophils and neutrophils in all further studies.

2.0 DEPENDENCE OF HUMAN GRANULOCYTE COMPLEMENT ROSETTES ON INCREASING CONCENTRATIONS OF C4 AND C3

As shown in Fig. 14 and Fig. 15, EAC14 and EAC1423 rosette formation by human eosinophils and neutrophils was directly related to the input of C4 or C3. Increasing amounts of these purified complement components resulted in increased numbers of eosinophil rosettes which paralleled the percentage of lysis obtained following the addition of the appropriate late components required to complete the haemolytic sequence. Similar results were obtained with neutrophils (not shown) although the percentage of rosetting cells was approximately two times greater. Therefore, the ability of eosinophils and neutrophils to recognise activated cell-bound human C4 and C3 can be demonstrated in a dose-dependent fashion.

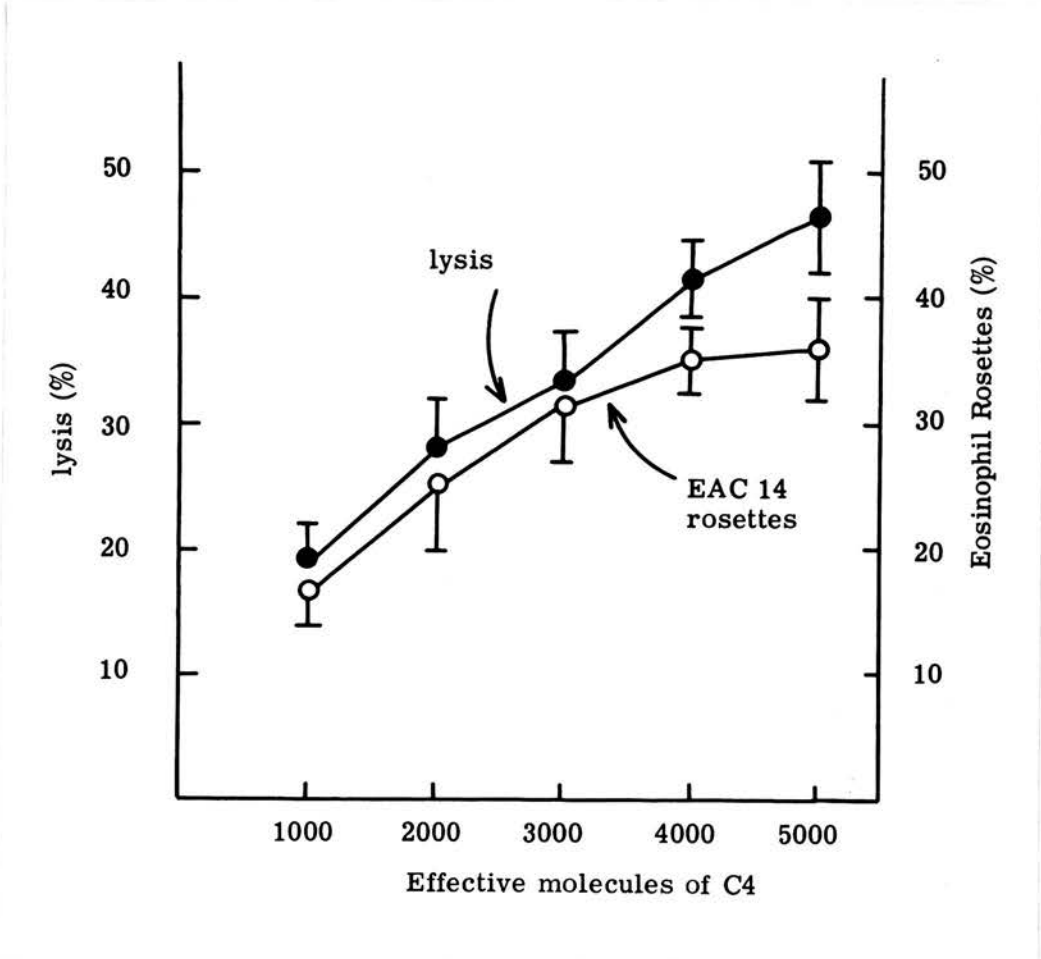

Fig. 14

The effect of increasing concentrations of human C4 on EAC14 eosinophil rosette formation and lysis with terminal human complement components (C2-C9).

The points represent the mean (± 1 S.D.) of five experiments.

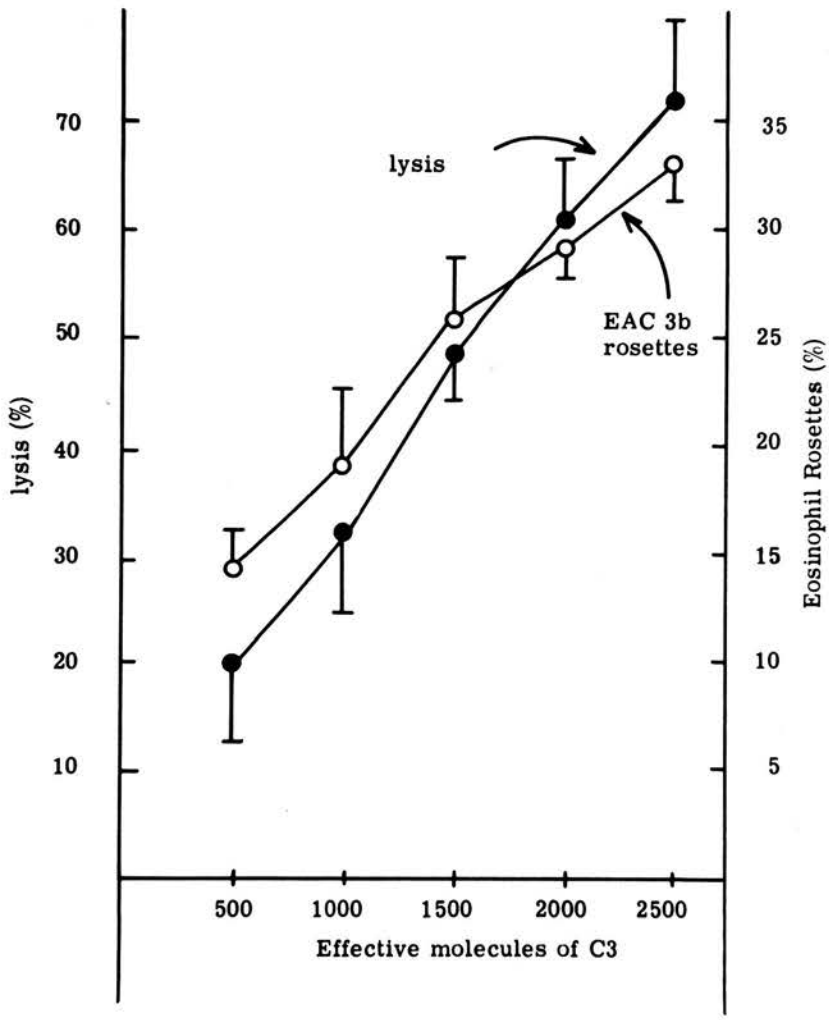

Fig. 15

The effect of increasing concentrations of human C3 on EAC3b eosinophil rosette formation and lysis with terminal human complement components (C5-C9).

The points represent the mean (± 1 S.D.) of five experiments.

Plate II

Eosinophil rosette formation with EAC14

(May-Grunwald/Giemsa, x 1000 magnification)

Plate III

Eosinophil rosette formation with EAC3b

(May-Grunwald/Giemsa, x 1000 magnification)

3.0 INHIBITION OF C4 ROSETTE FORMATION BY PURIFIED C2

It was observed during the course of these experiments that EAC142 cells did not form rosettes with either eosinophils or neutrophils. Knowing that C2 is a labile component, it was decided that this finding should be investigated in greater depth. In five experiments (Table I) it was found that C2 inhibited C4 rosette formation provided that the incubation was done at $0^{o}C$ so as not to allow the C2 to decay. Binding of EAC14 to the eosinophils and neutrophils can be restored by allowing C2 decay from EAC142 cells after incubation of EAC142 at $37^{o}C$ for 30 min, and the EAC142 can be shown to be in an EAC14 state since they can be agglutinated by monospecific anti-human C4. To show that the inhibition of EAC14 rosette formation was due to the bound C2 and not to the presence of C3b inactivator, which can also inactivate C4, the C2 preparation was assayed for the presence of C3b inactivator. It was found to be completely free from this enzyme. From these C2 decay experiments it was concluded that the addition of C2 to EAC14 may inhibit binding of C4 to eosinophils and neutrophils.

TABLE I

The effect of C2 decay on the binding of EAC142 with human eosinophils and neutrophils.

The C2 preparation was free of C3b inactivator.

PATIENT	EOSINOPHIL ROSETTES (%)		NEUTROPHIL ROSETTES (%)	
	$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$	$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$ (Decay)	$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$	$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$ (Decay)
C.D.	6.0	26.0	9.0	46.0
J.A.	8.0	27.0	8.5	53.0
M.B.	5.0	26.0	6.5	61.0
A.M.	4.0	22.0	6.0	55.0
J.N.	5.0	26.5	9.0	51.0

TABLE I

4.0 ROSETTE FORMATION BETWEEN EOSINOPHILS AND NEUTROPHILS AND EAC1423d

C3d coated red cells were prepared by treating EAC1423b cells with purified human C3b inactivator (KAF) for 1 hr at 37°C. These cells were referred to as C3d cells because they were, in contrast to the untreated C3b cells, no longer immune adherence positive, could not be lysed by the addition of C5-C9 and were more agglutinable by monospecific anti-C3d. It is now believed that the steps involved in the breakdown of C3b by the C3b inactivator are more complex (Harrison and Lachmann, 1978) (see Discussion).

Both EAC1423b and EAC1423d cells formed rosettes with eosinophils and neutrophils (Table II). However, the percentage of eosinophil C3d rosettes was significantly less than that for eosinophil C3b rosettes ($p < 0.05$). There was no difference between the percentage of C3b and C3d rosettes formed by neutrophils.

The percentage of eosinophils and neutrophils, from patients with eosinophilia, forming rosettes with EAC3d was similar when compared to cells from healthy individuals. Unfortunately only neutrophils and eosinophils from four individuals were tested for their capacity to form EAC3d rosettes (Table II).

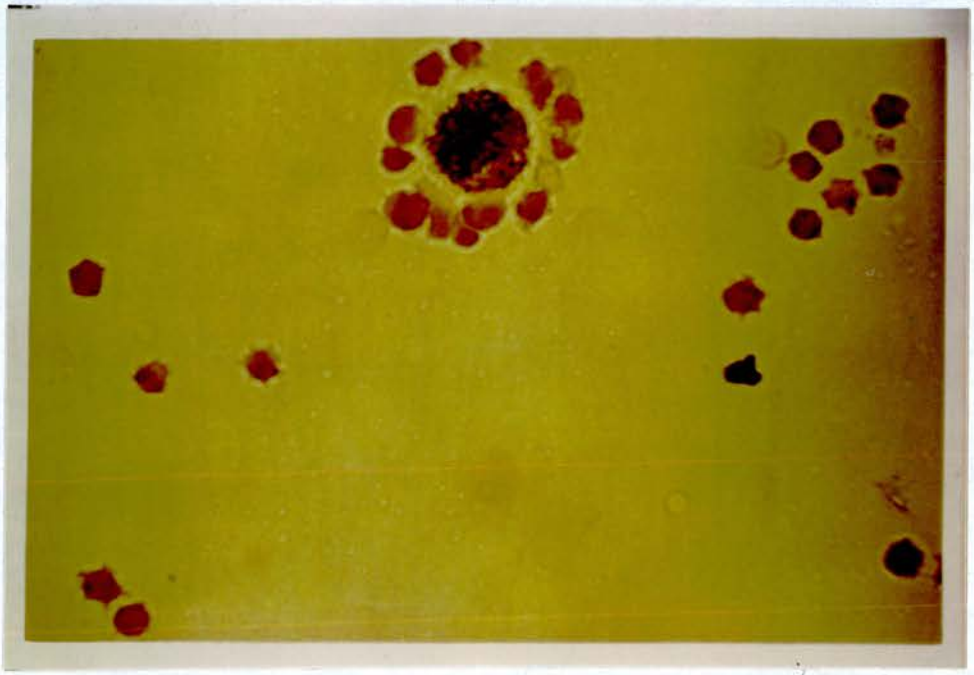

Plate IV

Eosinophil rosette formation with EAC3d

(May-Grunwald/Giemsa, x 1000 magnification)

TABLE II

Eosinophil and neutrophil rosettes with EAC3b and EAC3d.

Both EAC3b and EAC3d were prepared with 400 effective molecules of purified C4 per red cell.

PATIENT	DIAGNOSIS	EOSINOPHIL ROSETTES (%)		NEUTROPHIL ROSETTES (%)	
		EAC3b	EAC3d	EAC3b	EAC3d
J.C.	Unknown	33	25	60	63
E.B.	Pulmonary eosinophilia	33	23	62	62
M.H.	Lymphoma	37	24	59	56
D.F.	Atopic eczema	32	22	65	51
S.L.	Bronchial asthma	36	25	64	54
J.S.	Hypersensitivity to sulphonamides	33	24	68	53
A.M.	Bronchial asthma	35	20	63	54
A.W.	Atopic eczema	34	22	64	53
D.B.	Normal	43	26	62	58
P.D.	Normal	41	27	64	54
J.N.	Normal	38	24	65	52
G.R.	Normal	37	25	67	50

TABLE II

5.0 COMPARISON BETWEEN EOSINOPHILS AND NEUTROPHILS FROM PATIENTS WITH EOSINOPHILIA AND THOSE FROM HEALTHY DONORS

The percentages of eosinophils and neutrophils from 15 healthy individuals and 15 patients with eosinophilia of various aetiology forming rosettes with EAC14 or EAC1423 are shown in Fig. 7 and in Table V. The percentage of eosinophil rosettes with EAC14 or EAC1423 was significantly lower ($p < 0.05$) in the eosinophilic patients. When the neutrophils from the same patients were compared with neutrophils from the same 15 healthy donors, there was no significant difference in the percentage of rosetting cells with EAC14 or EAC1423 (Fig. 7). As with EA_G^{rab} rosettes, the percentage of blood eosinophilia and the percentage of rosetting eosinophils or neutrophils with EAC14 or EAC1423 in 15 eosinophilic patients were unrelated to the various disease states (Table V).

6.0 SUMMARY

The capacity of sheep red cells (E) coated with various purified human complement components to form rosettes with human neutrophils and eosinophils has been studied. The results are summarized in Table III. Untreated sheep red cells (E), E sensitized with rabbit IgM (EA_M^{rab}), EAC1 and EAC142 did not form rosettes with either neutrophils or eosinophils when tested at 0°C or at 37°C. The apparent rosette formation by EAC142 with both neutrophils and eosinophils at 37°C (shown in parenthesis in Table III) was thought to be due to C2 decay since the intermediate can then be shown to be in an EAC14 state rather than an EAC142 state as determined both antigenically and by haemolytic tests.

EAC14 (prepared with 4000 effective molecules of human C4 per red cell), EAC1423b and EAC1423d (both prepared with limited amounts of C4) formed rosettes with eosinophils and neutrophils. The rosette formation with both C3b and C3d was not tested at 0°C because it was shown from early experiments that complement rosettes are better expressed at 37°C.

Sheep red cells sensitized with rabbit IgG (EA_G^{rab}) formed rosettes with both eosinophils and neutrophils which, in contrast to complement rosettes, were better demonstrated at 0°C.

From these results it was concluded that human eosinophils and neutrophils bear membrane surface receptors for the human complement components C4, C3b and C3d. By decay experiments it was concluded that C2, in addition to

TABLE III

Eosinophil and neutrophil rosettes with untreated sheep red cells (E) or with E coated with antibody and various human complement intermediates.

The figures represent the mean values of 15 experiments (± 1 S.D.) with the exception of the C3d experiments which were performed eight times.

Rosette formation by EAC142 at 37°C was thought to represent EAC14 rosettes as a result of C2 decay and is, therefore, recorded in parenthesis.

EAC14 and EAC142 were prepared with 4000 effective molecules of C4 per cell.

EAC3b and EAC3d intermediates were prepared with 400 effective molecules of C4 per cell.

N.D. = not done.

INDICATOR CELLS	EOSINOPHIL ROSETTES (%)		NEUTROPHIL ROSETTES (%)	
	0°C	37°C	0°C	37°C
E	2.8 ± 1.0	5.1 ± 1.2	3.5 ± 2.0	6.2 ± 1.8
EA_G^{rab}	25.3 ± 3.6	16.3 ± 2.8	76.8 ± 7.6	58.3 ± 8.0
EA_M^{rab}	2.4 ± 1.2	3.3 ± 1.3	5.6 ± 2.2	4.5 ± 1.4
$EA_M^{rab}{}_{C1}{}^{hu}$	4.0 ± 1.5	6.0 ± 1.6	3.5 ± 1.0	4.6 ± 1.3
$EA_M^{rab}{}_{C1}{}^{hu}{}_4{}^{hu}$	10.0 ± 2.7	30.5 ± 1.8	54.8 ± 4.9	63.0 ± 7.5
$EA_M^{rab}{}_{C1}{}^{hu}{}_4{}^{hu}{}_2{}^{hu}$	5.2 ± 1.6	(15.5 ± 3.6)	7.0 ± 1.8	(42.5 ± 6.9)
$EA_M^{rab}{}_{C1}{}^{hu}{}_4{}^{hu}{}_2{}^{hu}{}_{3b}{}^{hu}$	N.D.	33.9 ± 2.4	N.D.	62.6 ± 2.6
$EA_M^{rab}{}_{C1}{}^{hu}{}_4{}^{hu}{}_2{}^{hu}{}_{3d}{}^{hu}$	N.D.	23.9 ± 1.6	N.D.	55.8 ± 4.4

TABLE III

TABLE IV

Eosinophil and neutrophil EA_G^{rab} rosette formation.

EA_G^{rab}				
PATIENT	DIAGNOSIS	EOSINOPHILIA (%)	EOSINOPHIL ROSETTES (%)	NEUTROPHIL ROSETTES (%)
D.N.	Bronchial asthma	10	22	76
J.R.	Carcinoma of the oesophagus	4	26	71
R.M.	Pulmonary eosinophilia	8	24	82
A.S.	Atopic eczema	12	32	81
I.C.	Urticaria of uncertain origin	21	29	88
J.B.	Pulmonary eosinophilia	21	28	77
H.A.	Schistosomiasis	10	26	69
E.M.	Bronchial asthma	10	25	70
A.H.	Bronchial asthma	11	21	82
C.A.	Atopic eczema	31	26	80
J.C.	Unknown	16	29	83
W.M.	Schistosomiasis	19	22	85
H.M.	Allergic vasculitis	10	26	78
H.C.	Atopic eczema	13	25	75
G.H.	Unknown	10	20	86

TABLE IV

TABLE V

Human eosinophil and neutrophil rosette formation with EAC14 and EAC3b.

$EA_M^{rab}C1^{hu}4^{hu}$					$EA_M^{rab}C1^{hu}4^{hu}2^{hu}3^{hu}$				
PATIENT	DIAGNOSIS	EOSINO-PHILIA (%)	EOSINOPHIL ROSETTES (%)	NEUTROPHIL ROSETTES (%)	PATIENT	DIAGNOSIS	EOSINO-PHILIA (%)	EOSINOPHIL ROSETTES (%)	NEUTROPHIL ROSETTES (%)
A.P.	Bronchial asthma	12	20	46	D.F.	Atopic eczema	14	32	65
K.M.	Bronchial asthma	14	23	55	D.M.	Atopic eczema	17	34	62
M.R.	Allergic rhini-tis	18	27	50	E.B.	Pulmonary eosinophilia	8	33	62
R.D.	Filariasis	13	24	48	M.H.	Lymphoma	52	37	59
H.W.	Bronchial asthma	11	23	52	S.L.	Bronchial asthma	13	35	63
C.A.	Atopic eczema	31	28	61	J.S.	Hypersensitivity to Sulphonamides	9	33	68
G.H.	Unknown	10	16	56	A.W.	Atopic eczema	12	34	64
J.B.	Pulmonary eosinophilia	21	18	50	J.C.	Unknown	16	33	68
H.A.	Schistosomiasis	10	18	48	E.J.	Bronchial asthma	14	35	62
R.B.	Filariasis	20	20	62	J.F.	Hypersensitivity to Carbimazole	17	35	64
H.M.	Allergic vasculitis	10	18	49	C.M.	Bronchial asthma	18	33	64
H.C.	Atopic eczema	13	25	48	M.B.	Bronchial asthma	13	35	63
A.H.	Bronchial asthma	11	17	55	B.D.	Bronchial asthma	21	30	58
M.R.	Pulmonary eosinophilia	16	23	53	J.S.	Hodgkin's disease	14	31	62
W.M.	Schistosomiasis	19	21	62	A.M.	Bronchial asthma	17	36	64

TABLE V

forming the classical pathway convertase with C1 and C4, inhibited eosinophil and neutrophil rosette formation with EAC14.

The percentage of eosinophils forming rosettes with EAC14 or EAC1423 was significantly reduced in patients with eosinophilia of various aetiology when compared to eosinophils from controls. When neutrophils from the same eosinophilic patients were compared to controls in terms of the percentage of EAC14 or EAC1423 rosettes, there was no significant difference.

These results, taken together with the previously known findings on the human monocyte, suggest that all circulating human phagocytic cells may have similar membrane receptors for promoting adherence of opsonized particles and that with eosinophils the numbers of complement receptors may be altered in disease.

SECTION III - ENHANCEMENT OF HUMAN EOSINOPHIL COMPLEMENT RECEPTORS BY PHARMACOLOGICAL MEDIATORS

SECTION III - CONTENTS

1.0 INTRODUCTION

An increase in the number of eosinophils, both in the tissues and circulation, is a characteristic feature of various clinical conditions of which common examples are diseases associated with immediate-type hypersensitivity. A considerable amount of information is now available on various mechanisms by which eosinophils accumulate at the site of allergic tissue reactions. For instance, various products of the anaphylactic reaction are preferentially chemotactic for eosinophils *in vitro*. These include the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay and Austen, 1971), now chemically characterised as two closely related acidic tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu) (Goetzl and Austen, 1975), histamine (Clark *et al*, 1975; Turnbull and Kay, 1976) and one of its major catabolites, imidazoleacetic acid (Turnbull and Kay, 1976).

At the present time, the exact mechanism by which eosinophils respond to these agents which cause random or directional motility is not clear although the participation of 'recognition units' present on the cell membrane has been suggested. It was proposed that the ECF-A peptides may interact with the eosinophil cell membrane through a receptor which has both a hydrophobic and ionic domain and to which the serine hydroxyl group of the valyl- or alanyl-peptide could attach by hydrogen bonding (Goetzl and Austen, 1976). In the earlier sections of this thesis the eosinophil cell membrane was shown to bear receptors for IgG and various complement components, C4, C3b and C3d. The relationship

between these receptors and those proposed for various chemotactic factors is unknown. Since the anaphylaxis-associated agents, such as ECF-A and histamine, are preferentially chemotactic for eosinophils, experiments described in this section were performed to see whether the pharmacological mediators of anaphylaxis have a direct effect on the eosinophil cell membrane, as assessed by their capacity to alter the expression of either IgG or complement receptors.

2.0 EFFECT OF INCREASING CONCENTRATIONS OF THE ECF-A PEPTIDES AND HISTAMINE

2.1 Effect on C3b and IgG receptors

In these experiments the leucocyte suspensions were incubated with increasing concentrations of the two ECF-A peptides or histamine (5×10^{-7}, 5×10^{-6} and 5×10^{-5} $mol.l^{-1}$), and they were added to the indicator sheep red cells to test their rosette forming capacity (see Materials and Methods). With increasing concentrations of the valyl-, alanyl-peptide or histamine there was a dose-dependent increase in the percentage of eosinophils, but not of neutrophils, which formed rosettes with EAC3b (Fig. 16). There was no increase in the percentage of eosinophils or neutrophils forming rosettes with EA_G^{rab}. Increasing concentrations of the valyl- or alanyl-peptide or histamine did not increase the percentage of monocyte EAC3b or EA_G^{rab} rosettes.

Further experiments were performed where eosinophils, neutrophils and monocytes were incubated with increasing concentrations of the peptides and histamine and then added to sheep red cells prepared with two doses of C3 (1000 and 2500 effective molecules per cell). The aim of these experiments was to exclude the possibility that by using a high dose of C3 on the indicator red cells (2500 molecules per cell), the neutrophils and monocytes, having more avid receptors for C3b, may express the maximum rosette formation and hence make the detection of any enhancement impossible. Dose-dependent enhancement of eosinophil rosette formation was observed with both doses of C3. The mean percentage

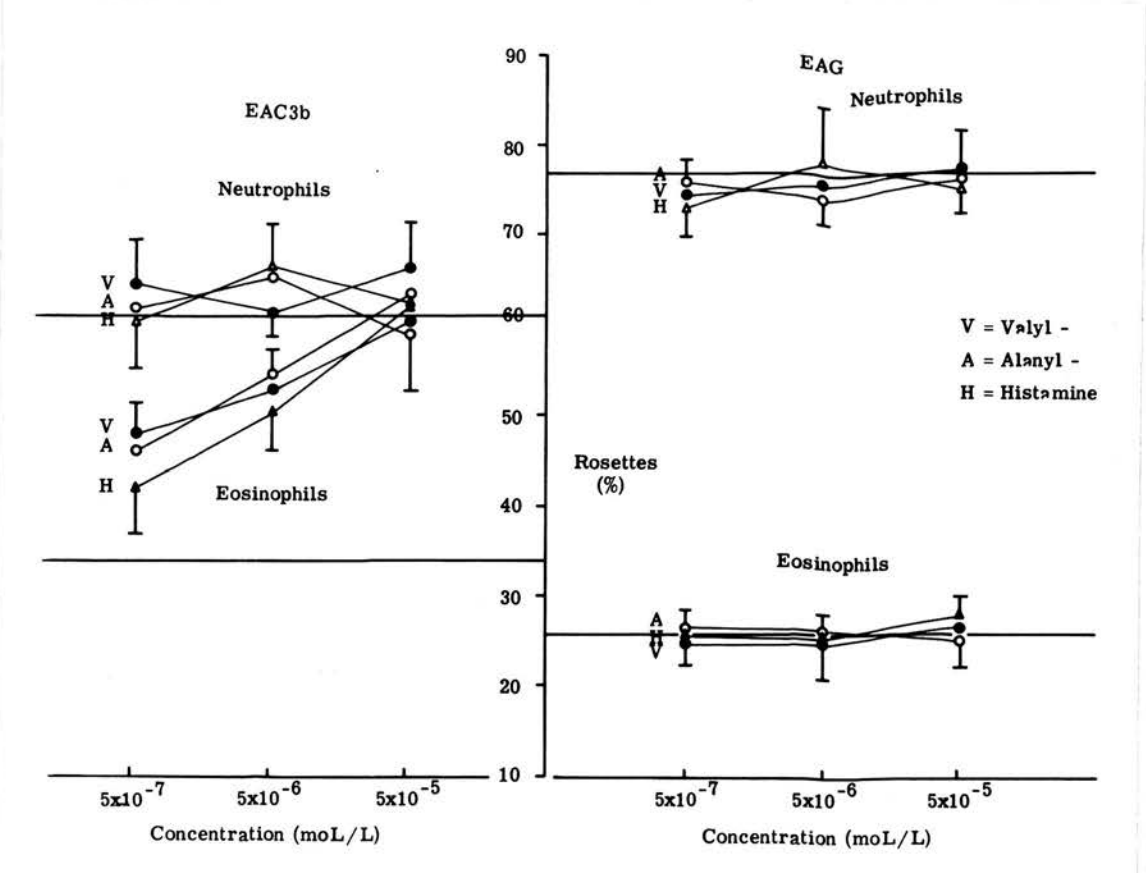

Fig. 16

The effect of increasing concentrations of the valyl- (●) and alanyl- (○) ECF-A peptides and histamine (▲) on EAC3b and EA_G^{rab} rosette formation by human eosinophils and neutrophils. The lines represent the percentage rosettes of untreated eosinophils and neutrophils.

Each point represents the mean (± 1 S.D.) of five experiments.

enhancement for EAC3b (containing 2500 effective C3 molecules/cell) rosettes at the highest concentration tested was 78% for the valyl-, 88% for the alanyl-peptide and 83% for histamine. When EAC3b cells containing less C3, i.e. 1000 effective molecules/cell, were used (so lowering the baseline percentage of rosettes) the percentage enhancement was 80%, 59% and 75% for the valyl-, alanyl-peptide and histamine respectively (Table VI). In contrast, neither ECF-A peptides nor histamine had any apparent effect on C3b rosette formation by neutrophils or monocytes, irrespective of whether the higher or lower dose of C3 per indicator red cell was used (Table VI).

In another series of experiments (not shown) the sensitizing IgG titre was lowered to give less neutrophil or monocyte EA_G^{rab} rosettes. Using these indicator red cells there was still no enhancement of EA_G^{rab} rosettes on either neutrophils or monocytes following their incubation with increasing concentrations of the peptides or histamine.

2.2 Effect on C4 and C3d receptors

Human eosinophils and neutrophils were shown to have membrane receptors for C4 and C3d (Section II). The effect of increasing concentrations of the ECF-A peptides and histamine on the C4 and C3d eosinophil receptors was also tested using sheep red cells coated with human C4 (EAC14) or with C3d (EAC1423d).

With increasing concentrations of either the valyl-, alanyl-peptide or histamine there was a dose-dependent increase in the enhancement of the EAC14 rosettes (Figs. 17a, 17b, 17c) which was very similar to the effect seen with

TABLE VI

The effect of the ECF-A tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu) and histamine on the percentage of eosinophil, neutrophil and monocyte EAC3b rosettes using EAC3b cells prepared with two concentrations of C3. The percentage enhancement is shown in parenthesis.

The figures represent the mean (± 1 S.D.) of five experiments (for monocytes tested with EAC3b prepared with 1000 molecules of C3 only three experiments were performed).

CONCENTRATION ($mol.l^{-1}$)	EOSINOPHILS		NEUTROPHILS		MONOCYTES	
	EFFECTIVE MOLECULES OF C3 PER CELL					
	2500	1000	2500	1000	2500	1000
UNTREATED	34 ± 3.0	22 ± 2.8	61 ± 4.5	36 ± 2.8	66.5 ± 2.8	46.5 ± 4.4
VAL-GLY-SER-GLU						
5×10^{-5}	60 ± 1.8 (78)	39 ± 4.1 (80)	66 ± 5.1 (7)	38 ± 2.2 (4)	63.5 ± 1.4 (-4.5)	44.7 ± 4.1 (-4)
5×10^{-6}	53 ± 2.0 (56)	32 ± 3.0 (47)	62 ± 2.6 (0.5)	41 ± 5.1 (12)	66.3 ± 1.8 (-0.3)	46.3 ± 1.4 (-0.4
5×10^{-7}	48 ± 2.8 (42)	30 ± 2.6 (36)	65 ± 4.8 (6)	35 ± 6.0 (-3)	62.0 ± 4.2 (-6.8)	46.6 ± 5.0 (0.2)
ALA-GLY-SER-GLU						
5×10^{-5}	64 ± 2.1 (88)	35 ± 3.5 (59)	59 ± 6.1 (-3)	36 ± 3.1 (-1)	61.8 ± 2.4 (-7)	47.5 ± 3.0 (2)
5×10^{-6}	54 ± 2.0 (61)	31 ± 2.6 (42)	66 ± 3.5 (7)	39 ± 2.5 (8)	65.3 ± 3.2 (-1.8)	44.3 ± 3.5 (-5)
5×10^{-7}	46 ± 7.0 (37)	29 ± 5.1 (31)	62 ± 2.2 (1)	41 ± 4.1 (13)	68.8 ± 1.8 (3.5)	45.3 ± 1.8 (-2.5
HISTAMINE						
5×10^{-5}	62 ± 7.2 (83)	38 ± 2.6 (75)	62 ± 3.8 (-0.3)	35 ± 3.2 (-5)	64.5 ± 3.5 (-3)	46.0 ± 5.3 (-1)
5×10^{-6}	50 ± 4.0 (49)	31 ± 4.3 (41)	66 ± 3.1 (8)	41 ± 6.1 (11)	64.3 ± 3.1 (-3.3)	48.5 ± 4.1 (4)
5×10^{-7}	42 ± 4.7 (25)	29 ± 2.9 (34)	61 ± 4.2 (-1)	38 ± 4.8 (5)	64.3 ± 6.0 (-3.3)	43.8 ± 1.5 (-6)

TABLE VI

EAC3b rosettes. The mean percentage enhancement for EAC14 rosettes, at the highest concentration tested, was 70 ± 8% for the valyl- and 71 ± 6% for the alanyl-peptide (Figs. 17a, 17b). With histamine the mean percentage enhancement for C4 rosettes was 65 ± 10% when tested at the highest dose (Fig. 17c). In contrast, the mean percentage enhancement for C3d rosettes using the same concentration of these agents was 18 ± 7% for the valyl-, 21 ± 8% for the alanyl-peptide and 17 ± 7% for histamine (Figs. 17a, 17b, 17c). These values represented an absolute increase from 28 ± 6% rosettes for the untreated controls to 33 ± 8% for the valyl-, 34 ± 6% for the alanyl-peptide and 33 ± 4% for histamine respectively, and were statistically insignificant.

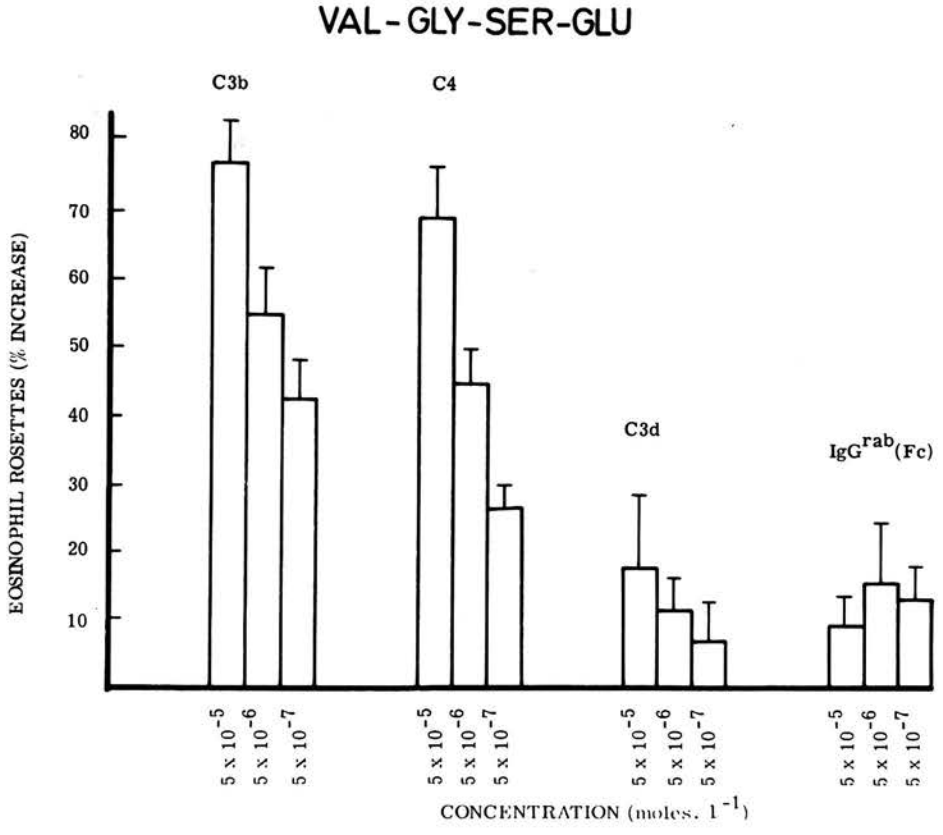

Fig. 17(a)

The effect of increasing concentrations of the valyl-peptide on eosinophil rosette formation with EAC3b, EAC14, EAC3d and EA_G^{rab}.

The bars represent the mean (± 1 S.D.) of five experiments.

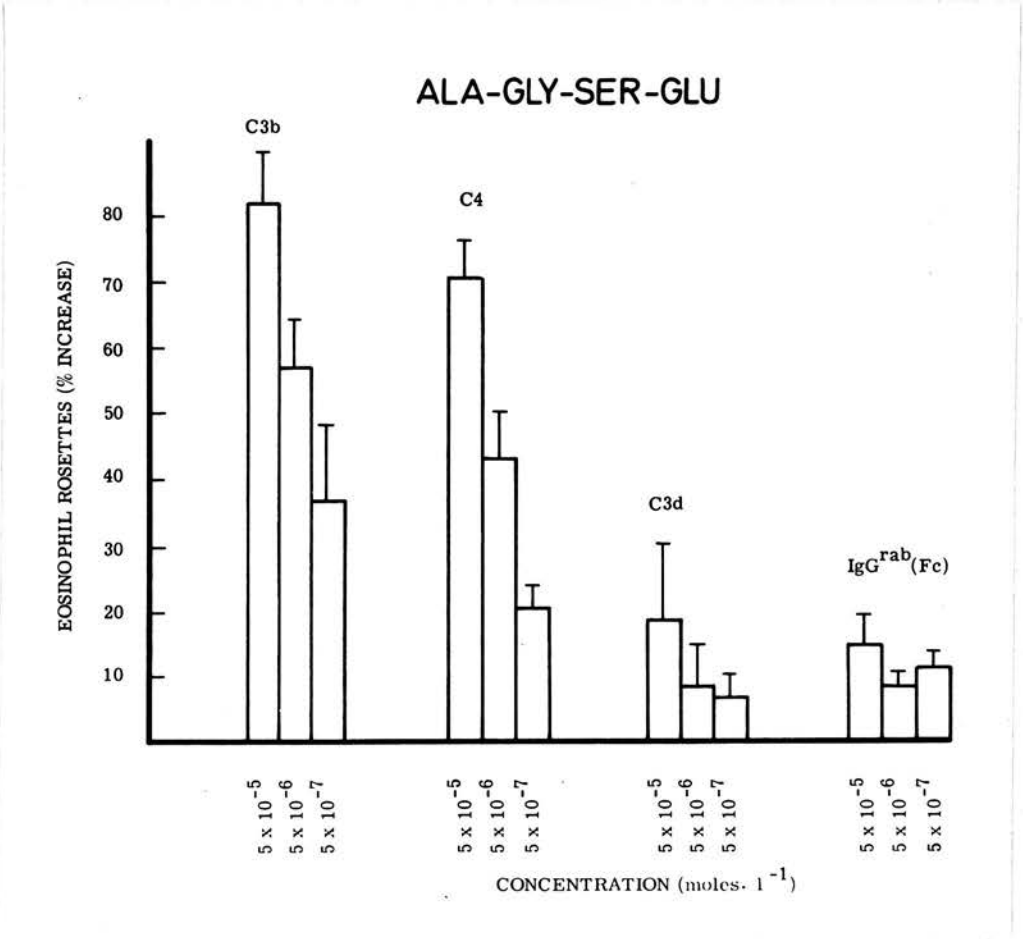

Fig. 17(b)

The effect of increasing concentrations of the alanyl-peptide on eosinophil rosette formation with EAC3b, EAC14, EAC3d and EA_G^{rab}.

The bars represent the mean (± 1 S.D.) of five experiments.

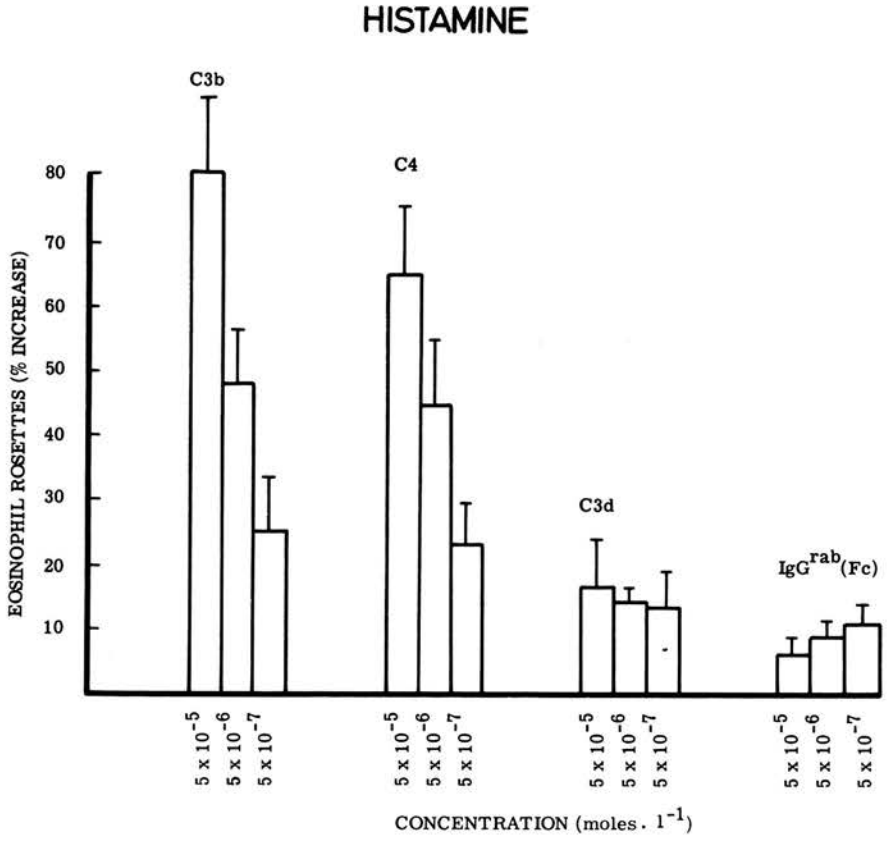

Fig. 17(c)

The effect of increasing concentrations of histamine on eosinophil rosette formation with EAC3b, EAC14, EAC3d and EA_G^{rab}.

The bars represent the mean (± 1 S.D.) of five experiments.

3.0 TIME-COURSE OF EOSINOPHIL COMPLEMENT RECEPTOR ENHANCEMENT

The enhancement of the eosinophil EAC3b rosette formation by both the ECF-A peptides or histamine was found to be time-dependent (Fig. 18). With the increase in the incubation time there was a corresponding linear increase in rosette formation by the valyl- and alanyl-peptide for the first 40 min after which their effect seemed to plateau. In contrast, the effect of histamine seemed to be delayed; most of the histamine-induced rosette enhancement occurring between 40 and 80 min.

The enhancement of EAC14 eosinophil rosettes by the ECF-A peptides with time gave virtually identical results to those observed for EAC3b. However, with histamine the enhancement of eosinophil EAC14 rosettes was similar to that observed with the ECF-A peptides, i.e. most of the increase being observed by 40 min (Fig. 19).

In subsequent experiments the time used to detect EAC3b and EAC14 eosinophil complement receptor enhancement was 60 min, i.e. well after the response had plateaued.

With C3d even when the incubation time was increased up to 180 min, no further increase was noticed in C3d eosinophil rosettes (Fig. 20). Similarly, there was no increase in IgG eosinophil rosettes following the incubation of the eosinophils with either of the ECF-A peptides or histamine for up to 3 hr.

In all these time-course experiments mentioned above the ECF-A peptides and histamine were tested at the highest concentration, i.e. 5×10^{-5} mol/l.

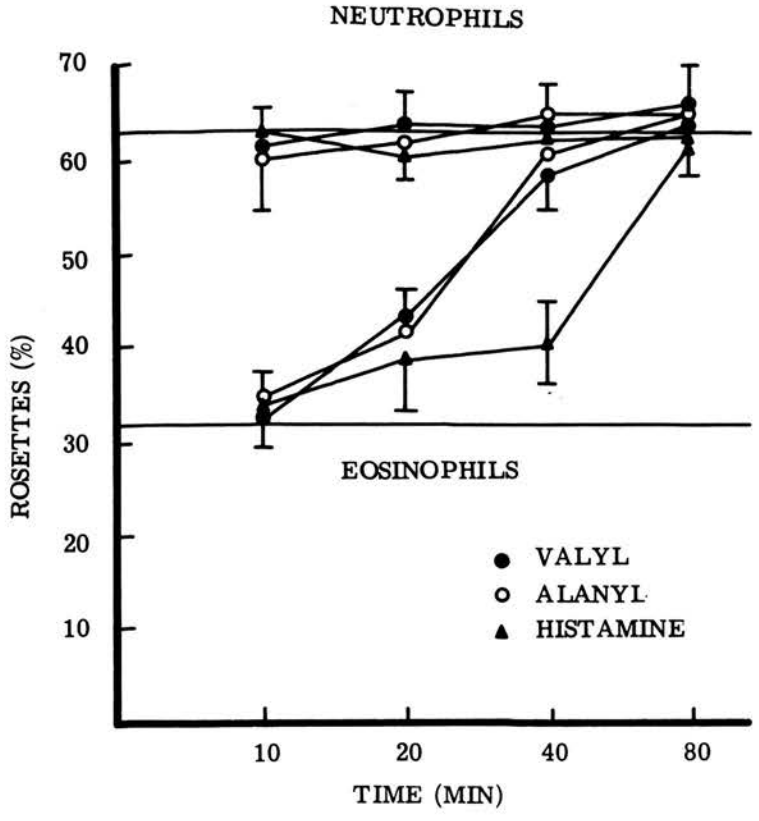

Fig. 18

The effect of time on enhancement of EAC3b rosette formation by eosinophils and neutrophils following incubation with the valyl- (●) and alanyl- (○) ECF-A peptides and histamine (▲).

Each mediator was tested at a concentration of 5×10^{-5} $mol.l^{-1}$.

The lines represent the percentage of rosettes of untreated eosinophils and neutrophils.

Each point represents the mean (± 1 S.D.) of five experiments.

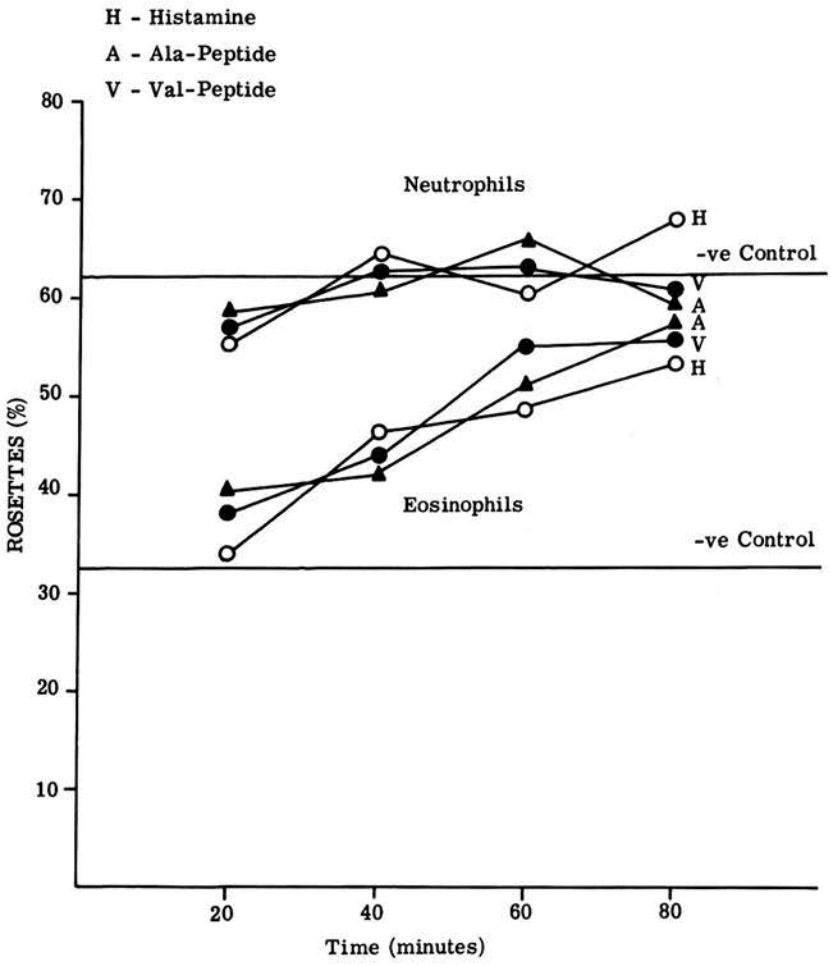

Fig. 19

The effect of time on enhancement of EAC14 rosette formation by human eosinophils and neutrophils following incubation with the valyl- (●) and alanyl- (▲) ECF-A peptides and histamine (O).

The mediators were tested at a concentration of 5×10^{-5} $mol.l^{-1}$.

The lines represent the percentage of rosettes of untreated eosinophils or neutrophils.

Each point represents the mean of two experiments.

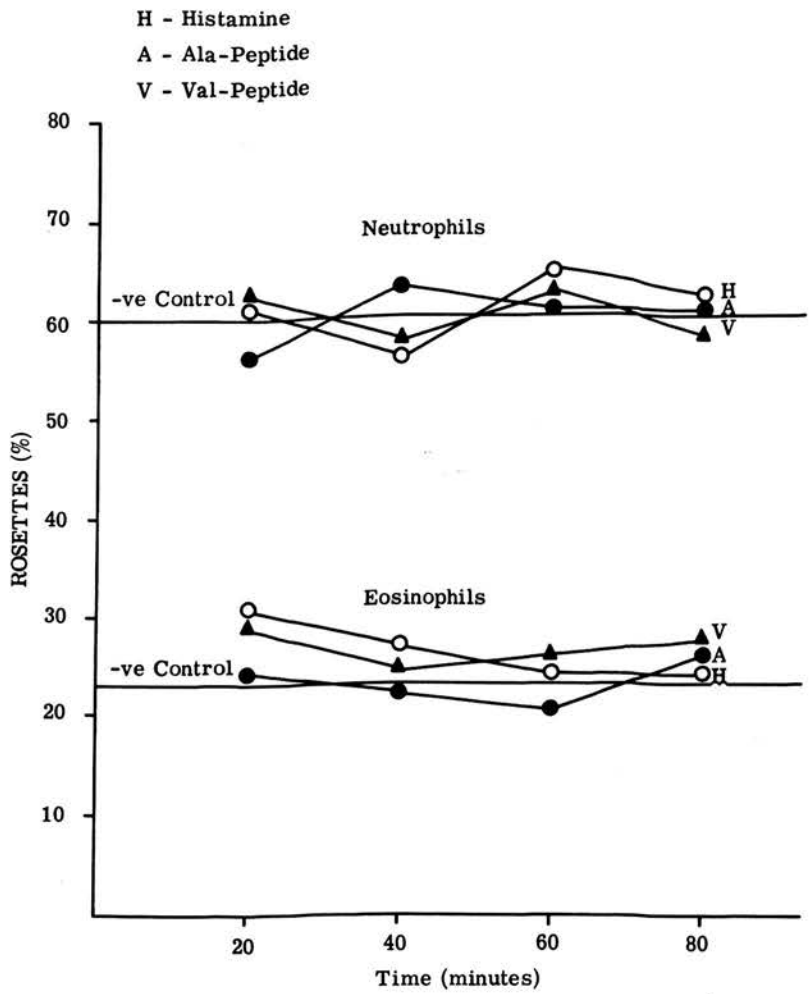

Fig. 20

The effect of time on enhancement of EAC3d rosette formation by human eosinophils and neutrophils following incubation with the valyl- (▲) and alanyl- (●) ECF-A peptides and histamine (O).

The three mediators were tested at a concentration of 5×10^{-5} mol.l^{-1}.

The lines represent the percentage of rosettes of untreated eosinophils or neutrophils.

Each point represents the mean of two experiments.

4.0 EFFECTS OF VARIOUS PRODUCTS OF THE ANAPHYLACTIC REACTION ON EOSINOPHIL COMPLEMENT RECEPTOR ENHANCEMENT

4.1 Effect of 'non-chemotactic' mediators

Together with histamine and ECF-A other mediators are known to be released during the anaphylactic reaction. These include bradykinin, 5-hydroxytryptamine and the prostaglandins PGE_1, E_2 and $F_{2\alpha}$. Experiments were performed in which these pharmacological mediators were incubated at comparable concentrations (5×10^{-5} to 5×10^{-7} mol/l) to those used for the ECF-A peptides or histamine to assess their effects on eosinophil C3b rosette forming capacity (Fig. 21). No significant enhancement was observed with bradykinin, PGE_1, PGE_2 or $PGF_{2\alpha}$. However, with 5-hydroxytryptamine an enhancement of 25 ± 2.6% was observed but only at the highest concentration tested (5×10^{-5} mol/l). The enhancement of histamine or with the ECF-A peptides was almost three times this value.

4.2 Effect of a human anaphylactic lung diffusate

A human lung anaphylactic diffusate (containing a final histamine concentration of 8.1×10^{-6} mol/l) gave an enhancement of C3b rosettes in a dose-dependent fashion (Fig. 21). The appropriate controls, i.e. diffusates from unsensitized lung challenged with the antigen (TGP) or sensitized lung fragments incubated only with Tyrode's buffer, gave no significant enhancement. A 1 in 10 dilution of the anaphylactic diffusate (containing a final histamine concentration of 4.05×10^{-7} mol/l) gave a C3b rosette enhancement of 48 ± 4.5%. With histamine alone, a

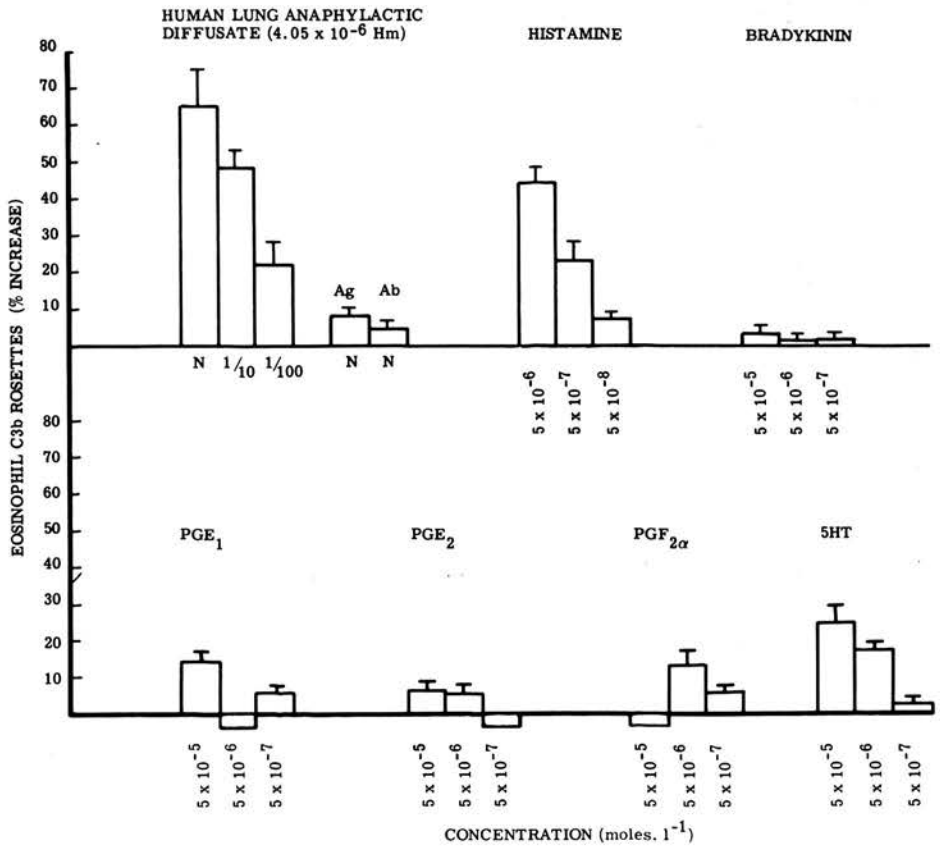

Fig. 21

The effect of a human anaphylactic lung diffusate and various synthetic pharmacological mediators of anaphylaxis on EAC3b rosette formation by human eosinophils.

The bars represent the mean (± 1 S.D.) of five experiments (with the exception of the diffusate and bradykinin - three experiments).

comparable enhancement was only achieved with a dose of 5×10^{-6} mol/l, that is about ten times more histamine than that contained in the anaphylactic diffusate.

4.3 Effect of mixtures of ECF-A peptides and histamine on eosinophil complement receptor enhancement

Since the anaphylactic lung diffusate is known to contain the ECF-A tetrapeptides in addition to histamine and other pharmacological mediators, experiments were performed to see whether there was an additive or synergistic effect, in terms of complement receptor enhancement, when synthetic peptides and histamine were mixed in various combinations. When 5×10^{-5} mol/l of valyl- or alanyl-peptide were mixed with histamine over the dose range 5×10^{-5} to 5×10^{-7} mol/l the final enhancement of the eosinophil C3b rosettes was either slightly (but insignificantly) greater, or the same as that observed when each agent was tested separately (Fig. 22a, 22b).

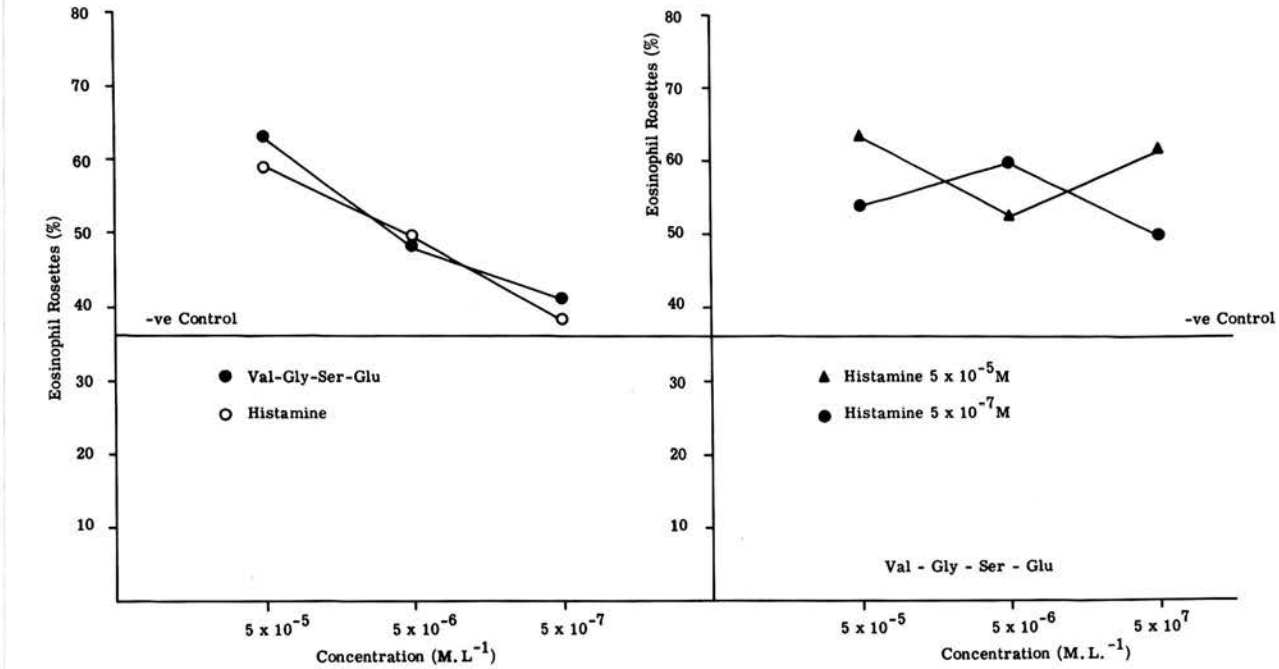

Fig. 22(a)

The effect of mixing histamine at two concentrations (5×10^{-5}, 5×10^{-7} $mol.l^{-1}$) with increasing concentrations of the valyl-peptide on the enhancement of EAC3b rosette formation by human eosinophils.

The lines represent the percentage of rosettes with untreated eosinophils.

The points represent the mean of two experiments.

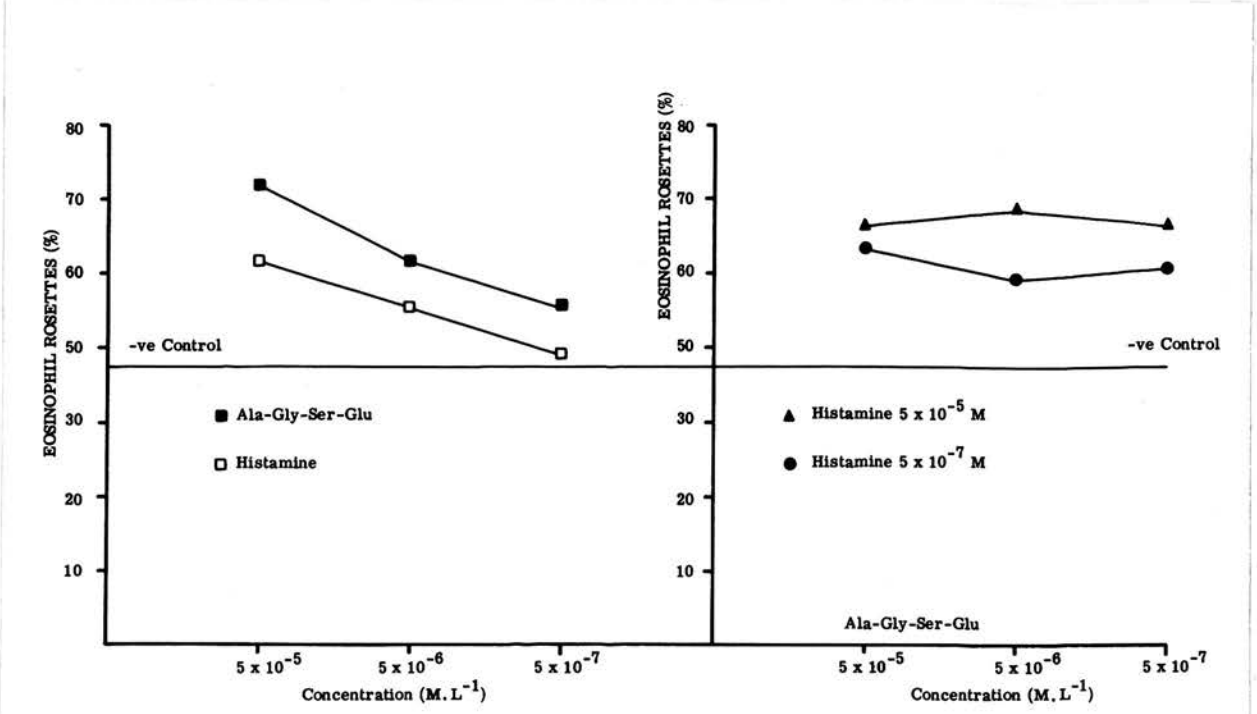

Fig. 22(b)

The effect of mixing histamine at two concentrations (5×10^{-5}, 5×10^{-7} mol.l^{-1}) with increasing concentrations of the alanyl-peptide on the enhancement of EAC3b rosette formation by human eosinophils.

The lines represent the percentage of rosettes with untreated eosinophils.

The points represent the mean of two experiments.

5.0 EFFECT OF A HISTAMINE PRECURSOR (HISTIDINE) AND SOME MAJOR HISTAMINE CATABOLITES ON EOSINOPHIL COMPLEMENT RECEPTOR ENHANCEMENT

L-histidine (a histamine precursor) and some of its major catabolites (imidazoleacetic acid (ImAA), 1,4-methylhistamine (1,4-MeHm), 1,4-methyl-imidazoleacetic acid (1,4-MeImAA) and N-acetylhistamine (N-AcHm)) were tested, at comparable concentrations to those used for the ECF-A tetrapeptides or histamine, for C3b receptor enhancing activity. It can be seen from Fig. 20 that enhancement of eosinophil C3b rosettes was only observed with imidazole-acetic acid, which has previously been reported to be a selective chemoattractant for human eosinophils. The enhancement produced by ImAA was similar to that for histamine in terms of the concentrations used and also in terms of the magnitude of the dose-response (Fig. 23).

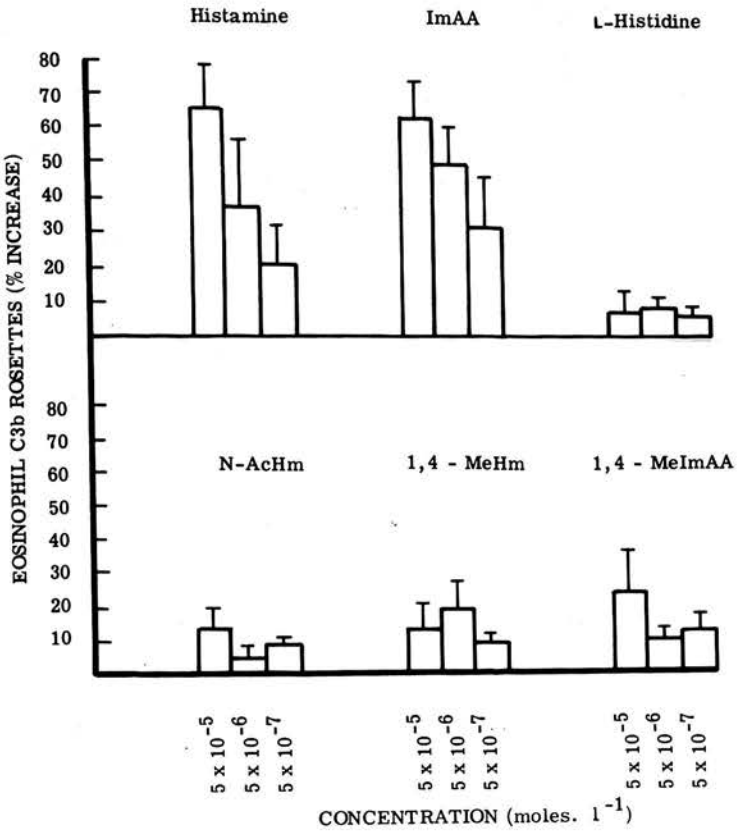

Fig. 23

The effect of various concentrations of histamine, L-histidine and the major histamine catabolites on eosinophil EAC3b rosette formation.

The bars represent the mean (± 1 S.D.) of five experiments.

6.0 SUMMARY

The ECF-A tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu) and histamine markedly enhanced the expression of human eosinophil receptors for C3b. The enhancement was both dose- and time-dependent, and highly selective for the eosinophil since there was no evidence that C3b receptors on neutrophils or monocytes were altered by these pharmacological agents even when varying concentrations of C3 were added to the indicator red cells.

The agents similarly enhanced receptors for C4 but under the same conditions C3d and IgG receptors were unaffected.

A number of other pharmacological mediators including bradykinin, and the prostaglandins PGE_1, E_2 and $F_{2\alpha}$ had no apparent effect on eosinophil C3b receptors at the same molar concentrations (5×10^{-5} to 5×10^{-7} mol/l). However, a major histamine catabolite, imidazoleacetic acid, also recognised as an anaphylaxis-associated eosinophilotactic agent, enhanced eosinophil C3b receptors to a degree comparable to that of histamine. In contrast, L-histidine and the histamine catabolites, N-acetylhistamine, 1,4-methylhistamine and 1-methyl-4-imidazoleacetic acid, had no eosinophil C3b enhancing effect. 5-hydroxytryptamine, at the highest concentration tested (5×10^{-5} mol/l), gave enhancement of eosinophil C3b receptors which was approximately one-third of that achieved with the same concentration of the ECF-A peptides, histamine or imidazoleacetic acid.

A human lung anaphylactic diffusate also enhanced eosinophil C3b receptors. Approximately ten times more

synthetic histamine than that contained in the lung-derived material was required for comparable enhancement indicating that the histamine and ECF-A peptides present in the diffusate may have combined to give an optimal enhancing effect. However, when synthetic histamine and the valyl- or alanyl-peptide were mixed in various proportions, the enhancing effect was no greater than that achieved when each agent was tested alone.

These results may suggest that pharmacological mediators of hypersensitivity may regulate certain eosinophil-dependent biological reactions and that there may be a direct relationship between the eosinophil surface 'recognition unit' for eosinophil migration and some of the membrane receptors which promote the adhesion of the eosinophil to opsonized particles.

SECTION IV - THE PARTICIPATION OF ANTIBODY AND/OR COMPLEMENT IN EOSINOPHIL-DEPENDENT KILLING OF SCHISTOSOMULA OF SCHISTOSOMA MANSONI

SECTION IV - CONTENTS

1.0 INTRODUCTION

For many years an association between helminth infection and increased levels of peripheral blood and tissue eosinophils has been recognised. In human disease this association is particularly marked in patients with filariasis, schistosomiasis, ascariasis, trichinosis and visceral larva migrans. In addition, tropical pulmonary eosinophilia is now believed to be associated with cryptic filarial infections (Neva et al, 1975). In animals infected with helminths such as Fasciola hepatica and Oesophagostomum radiatum high levels of blood eosinophils are also a common finding. In experimental animals infection with Trichinella spiralis is one of the most reliable sources of inducing a peripheral blood eosinophilia.

In spite of this consistent and striking relationship between eosinophilia and helminth infection, it was not easy to attribute a specific functional role to the eosinophil in such situations. However, more recently several workers have described various in vitro and in vivo cell-mediated effector systems in which eosinophils and other leucocytes may be involved in limiting the effects of helminth infections. Dean et al (1974) demonstrated a complement-dependent IgG antibody in immune rats and guinea pigs (1975) which would rapidly cause damage to the larval stage (schistosomulum) of Schistosoma mansoni in the presence of neutrophils in vitro. This damage was associated with discharge of neutrophil enzymes on to the surface of the organisms.

In 1974 Perez described a macrophage-dependent reaction

capable of damaging schistosomula which was dependent on a heat-stable cytophilic IgG antibody which confers on macrophages the ability to adhere and kill schistosomula. Capron et al (1975) described another macrophage-dependent system in which normal rat macrophages can adhere and kill schistosomula if they were preincubated with a heat-labile factor in immune rat serum. This factor was later found to be an IgE antibody.

More recently the human eosinophil was shown to mediate antibody-dependent damage of schistosomula of Schistosoma mansoni in vitro (Butterworth et al, 1975). In this system larval damage was measured by the release of ^{51}chromium from pre-labelled schistosomula and the antibody involved was shown to be IgG (Butterworth et al, 1977b). Similar antibody-dependent eosinophil damage to schistosomula has subsequently been described in the baboon (Butterworth et al, 1976) and in rat (Mackenzie et al, 1977).

In 1978 Ramalho-Pinto et al found that schistosomula can be coated with C3 in the absence of antibody through the activation, by the tegument, of the alternate pathway of the complement system following their incubation with fresh rat serum. These schistosomula, coated with C3, were shown to be more susceptible to damage by rat eosinophils than IgG coated organisms.

The experiments described in this section were designed to study the capacity of the human eosinophil to mediate damage to schistosomula coated with antibody and/or complement. Also, since rat neutrophils and macrophages were previously implicated in damage to schistosomula, human neutrophils and

mononuclear leucocytes were compared with eosinophils in terms of their capacity to kill schistosomula. Other experiments were performed to see whether schistosomula can activate human complement by the alternate pathway. Also the role played by combining both human antibody and complement in schistosomula damage by various human leucocytes, which is presumably more relevant to the *in vivo* situation, was assessed.

2.0 THE ROLE OF ANTIBODY AND/OR COMPLEMENT

In the eight experiments shown in Table VII granulocyte preparations obtained from eight eosinophilic patients were tested for their capacity to mediate schistosomula (Sch) damage in three different experimental systems, i.e. antibody alone (Ab), complement alone (C), and a combination of antibody and complement (Ab + C). The granulocyte preparations (G) contained only eosinophils or neutrophils, contamination by other cell types being less than 3%. Also, mononuclear cells (M) from the same patients were examined as regards their capacity to cause schistosomula damage in the three different systems. With granulocytes, killing with antibody alone (Sch + Ab + G) was approximately two and one half times greater than the appropriate control (Sch + G). Granulocyte killing of schistosomula coated with complement via the alternate pathway activation (Sch + C + G) was about four times greater than the appropriate control (Sch + C^H + G) and about two times greater than killing with antibody alone. However, it is clear from this table that the most efficient system, under these experimental conditions, was the combination of antibody and complement (Sch + Ab + C + G). The relative efficiencies of the three systems expressed in terms of the mean ± 1 S.D. percentage killing for the eight experiments shown were 70 ± 8 for Sch + Ab + C + G, 52 ± 9 for Sch + C + G and 31 ± 7 for Sch + Ab + G.

From these experiments there was no evidence that there was an association between the percentage of eosinophils in the granulocyte cell suspensions and the magnitude of the damage to the schistosomula.

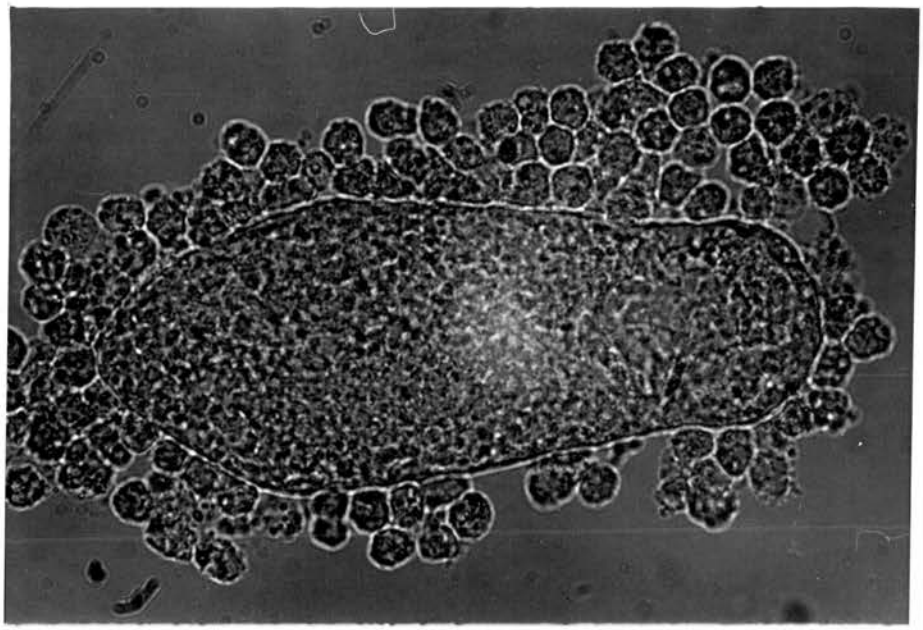

Plate V

Adherence of granulocytes to schistosomula coated with complement. Note the intact tegument. (x 400 magnification)

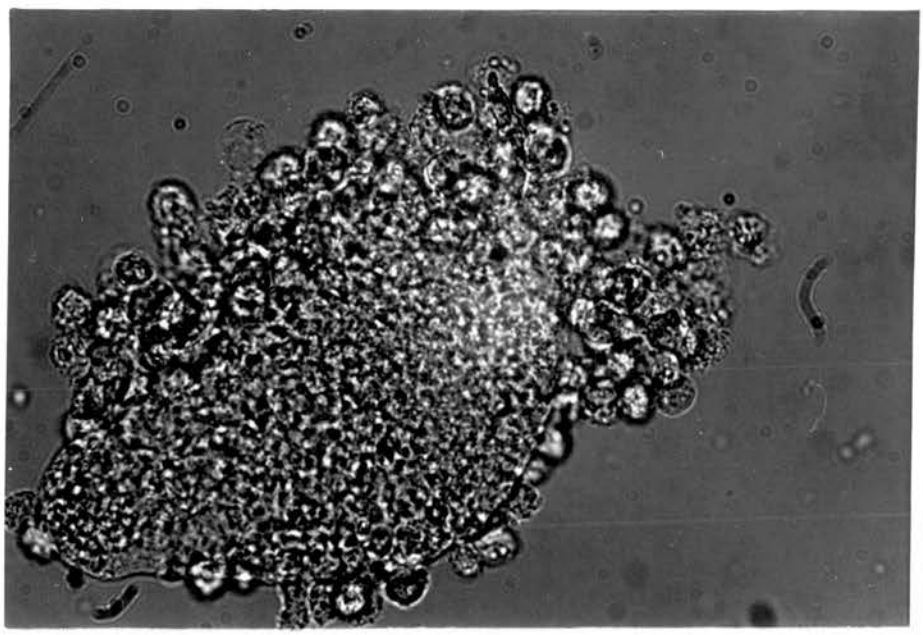

Plate VI

Granulocyte adherence and killing of schistosomula coated with complement. Note the damaged tegument on one side of the worm.

(x 400 magnification)

TABLE VII

Killing of schistosomula (Sch) coated with antibody alone 'Ab alone', complement alone 'C alone' and antibody + complement 'Ab + C' by: (a) granulocytes (G) of varying eosinophil concentrations, (b) mononuclear leucocytes (M).

The figures represent the percentage killing for each duplicate well.

C^H = heat inactivated autologous serum.

EXPERIMENT NO.	1	2	3	4	5	6	7	8	Mean ± S.D
A. GRANULOCYTES									
Eosinophils (%) in granulocyte suspension	15	17	53	55	58	63	66	72	
Sch + Ab + G	44, 40	32, 25	33, 27	36, 27	18, 26	27, 25	38, 31	30, 34	30.8 ± 6.8
Sch + C + G	52, 56	50, 43	55, 45	46, 40	40, 47	55, 57	53, 61	68, 68	52.2 ± 8.8
Sch + Ab + C + G	70, 78	59, 52	66, 73	70, 66	-	67, 76	66, 75	77, 78	69.5 ± 8.4
Sch + C^H + G	18, 17	12, 18	15, 9	10, 14	17, 10	12, 11	14, 20	19, 15	14.4 ± 3.3
Sch + G	14, 18	10, 12	15, 11	17, 13	16, 11	6, 4	13, 17	10, 13	12.5 ± 3.7
B. MONONUCLEAR LEUCOCYTES									
Sch + Ab + M	20, 22	26, 20	26, 23	26, 20	19, 16	22, 18	21, 27	20, 22	21.8 ± 3.1
Sch + C + M	34, 30	38, 29	34, 34	28, 31	24, 21	31, 37	32, 26	34, 30	30.8 ± 4.5
Sch + Ab + C + M	37, 33	36, 44	33, 40	37, 40	-	42, 33	32, 40	33, 37	36.9 ± 3.8
Sch + C^H + M	13, 18	17, 23	16, 19	14, 20	13, 13	9, 16	22, 18	13, 18	16.3 ± 3.6
Sch + M	8, 11	13, 7	8, 6	9, 11	7, 10	10, 5	12, 10	8, 11	9.1 ± 2.2
NO LEUCOCYTES									
Sch + C + Ab	10, 8	-	-	14, 10	13, 6	17, 10	13, 14	9, 15	11.6 ± 3.1
Sch + 199	6, 9	-	-	13, 8	11, 4	14, 8	10, 10	6, 12	9.3 ± 2.9

TABLE VII

Damage of schistosomula by mononuclear cells was significantly lower ($p < 0.001$) in all three systems when compared to equivalent damage by granulocytes (Table VII), although it remained markedly higher than the appropriate control. The mean ± 1 S.D. percentage killing by mononuclear cells was 37 ± 4 for Sch + Ab + C, 31 ± 5 for Sch + C, and 22 ± 3 for Sch + Ab.

2.1 Effect of increasing concentrations of antibody and complement

When various dilutions of fresh normal human serum (as a source of complement) were used, the percentage of schistosomula killed both in the Ab + C system (using a constant amount of antibody) and with 'C alone', was directly related to the concentration of the serum used. Similarly, dilutions of human anti-schistosomiasis serum used in the 'Ab alone' system resulted in a decrease in the percentage of schistosomula killed (Fig. 24). Essentially the same results were found with the mononuclear cells.

2.2 Participation of purified human IgG

Human IgG was purified from whole anti-schistosomiasis serum by ion exchange chromatography on a DE-52 column (see Methods). In two different experiments illustrated in Fig. 25 it was shown that when this purified IgG was used instead of the whole antiserum there was very little difference in terms of the percentage of schistosomula killing in both the 'Ab alone' and the 'Ab + C' systems. These results suggested that IgG might have accounted for virtually all the activity

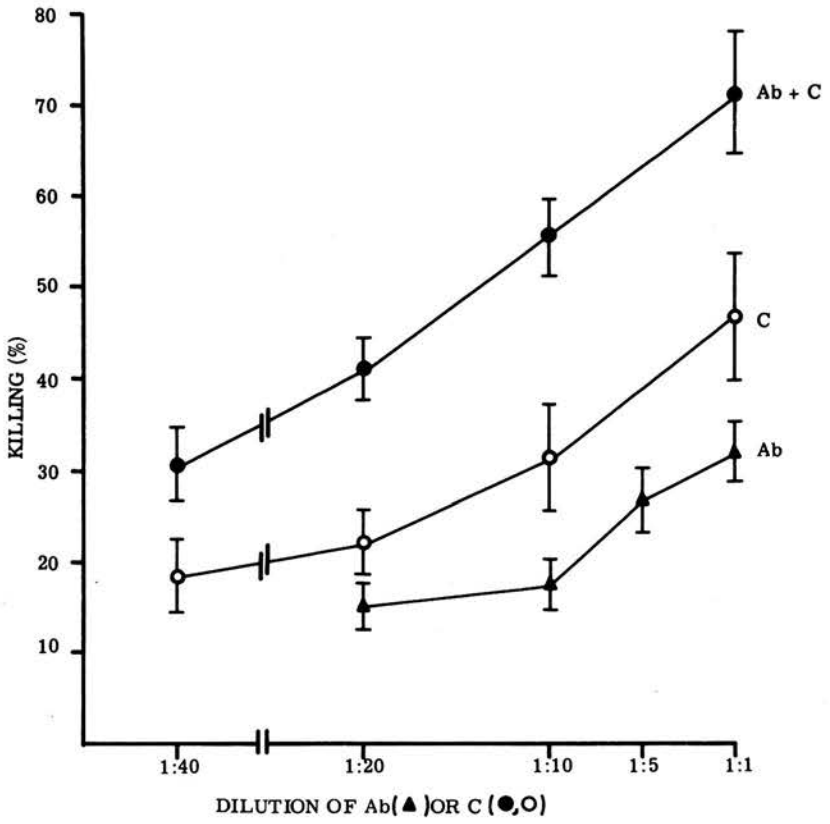

Fig. 24

The effect of varying the concentrations of antibody or complement on the granulocyte killing of schistosomula coated with antibody and/or complememt.

The points represent the mean (± 1 S.D.) of four experiments.

present in the anti-schistosomiasis serum. Further evidence was obtained when a fraction eluted from the DE-52, which contained no IgG but an equivalent amount of IgA and IgM to those present in the whole anti-schistosomiasis serum, was tested for its capacity to damage schistosomula, and shown to give a percentage killing no greater than that obtained with controls (Fig. 25).

2.3 (a) Participation of the classical pathway of the complement system

In these experiments human purified complement components were used. To sensitized schistosomula (SchA) the early classical complement components, i.e. C1, C4, C2 and C3, were added sequentially to form SchAC1423. The presence of schistosomula-bound C3 was confirmed directly by immunofluorescence using fluorescein-labelled anti-human C3 (Table VIII). The Sch AC1423 were found to be susceptible to granulocyte killing. Furthermore, the addition of C3 to SchAC142 resulted in a proportional increase in the percentage killing of schistosomula by granulocytes which was directly related to the number of effective molecules of C3 added per schistosomulum (Fig. 26). However, schistosomula coated with (1) antibody alone (SchA), (2) C1 and C4 (SchAC14) or (3) C1, C4 and C2 (SchAC142), were all equally susceptible to killing by granulocytes.

2.3 (b) Participation of the alternate pathway of the complement system

Following the incubation of schistosomula with fresh normal human serum the tegument of the schistosomula can be

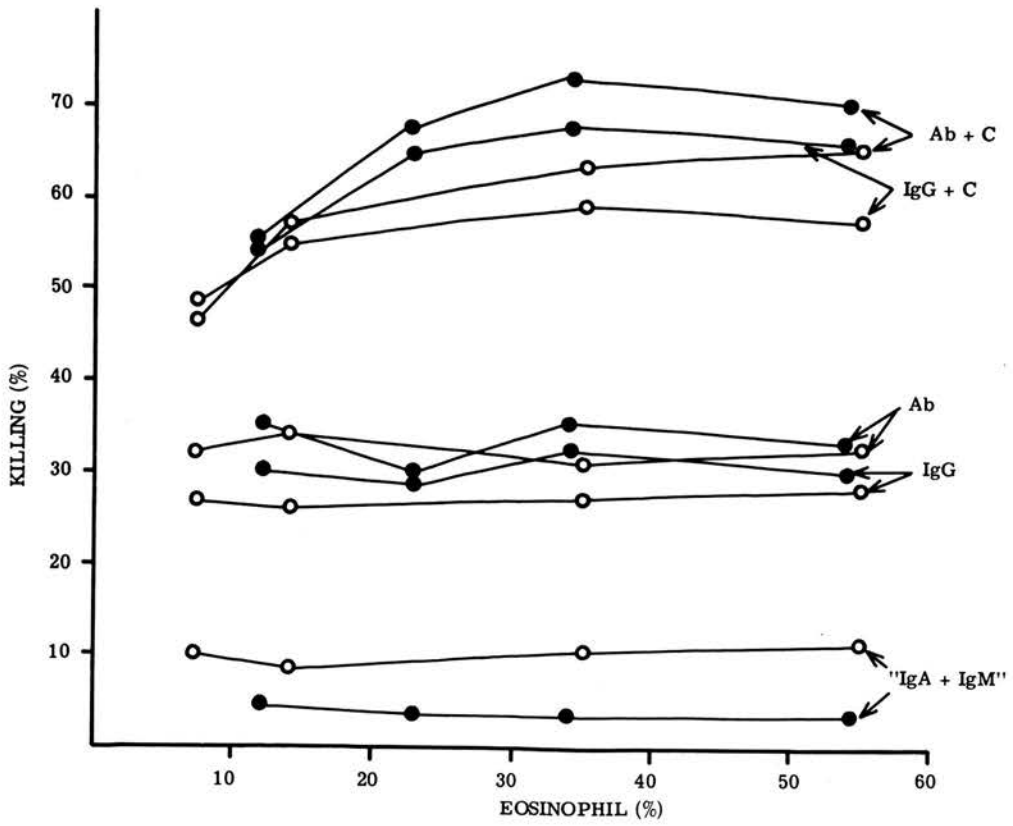

Fig. 25

The effect of fractionated IgG and unfractionated (Ab) anti-schistosomula sera on schistosomula killing, with or without complement, by granulocyte suspensions containing increasing eosinophil concentrations.

The effect of DE-52 fraction containing an equivalent amount of IgA and IgM to that present in the unfractionated serum is also shown.

Two different experiments (● and O) are shown.

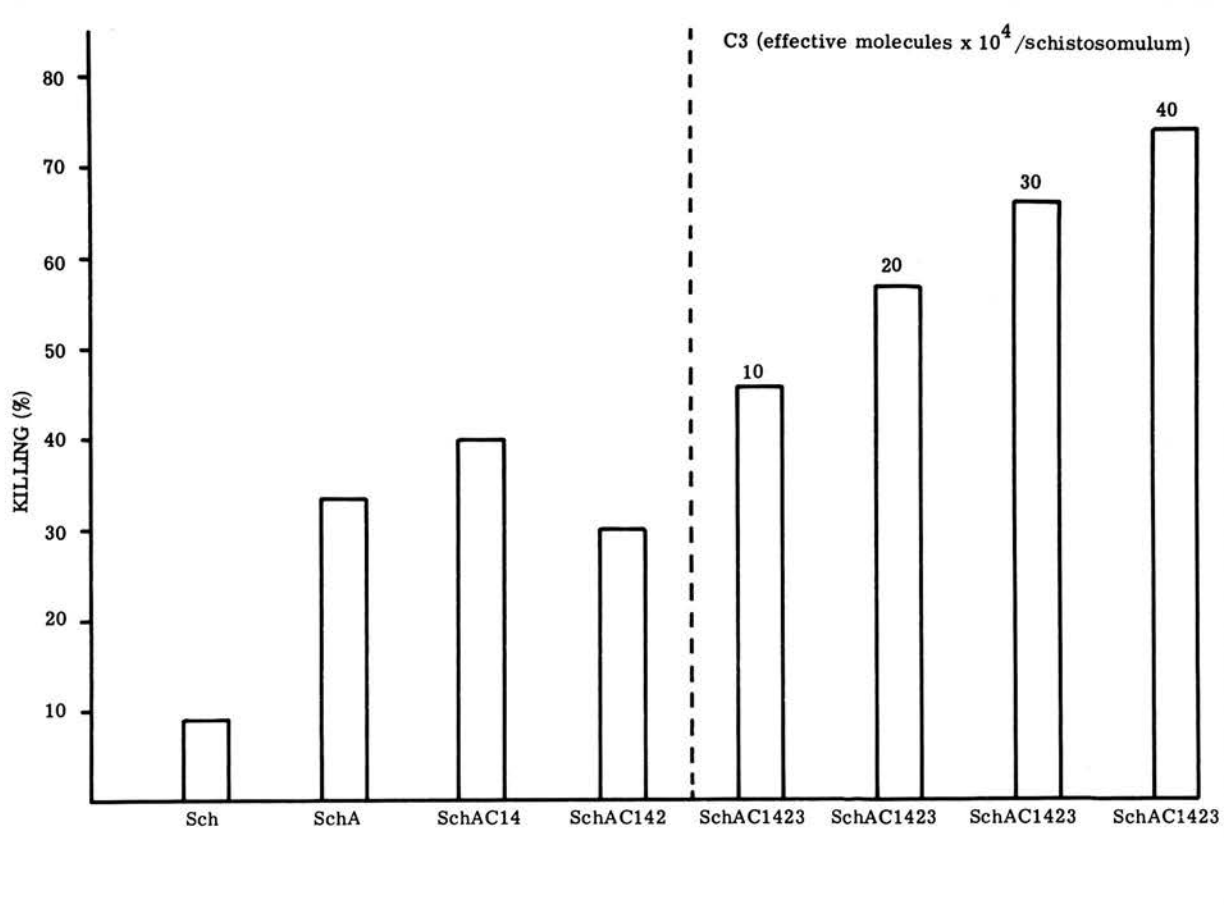

Fig. 26

The killing of schistosomula (Sch) coated with human anti-schistosomula serum (A) and purified components of the classical pathway of human complement.

Increasing concentrations of purified human C3 were added to SchAC142 (to the right of the interrupted line).

The columns represent the mean of three experiments.

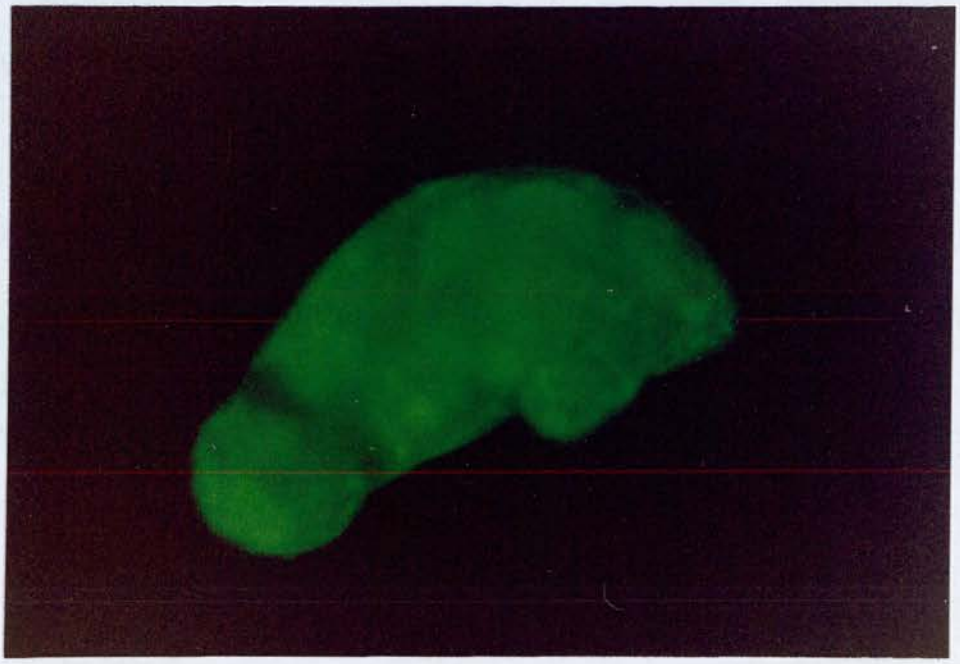

Plate VII

Fluorescence of schistosomula coated with C3 (through classical pathway activation, i.e. sensitized schistosomula + C1423) in the presence of fluorescent labelled anti-human C3. (x 400 magnification)

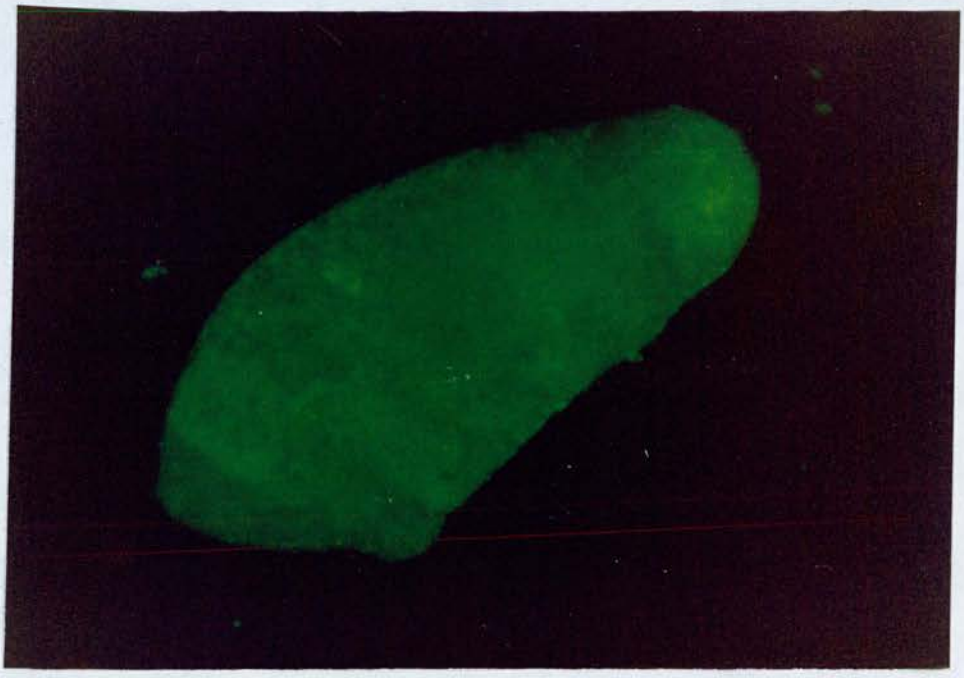

Plate VIII

Fluorescence of schistosomula coated with C3 (through alternate pathway activation) in the presence of fluorescent labelled anti-human C3. (x 400 magnification)

shown to be coated with large amounts of C3 using fluorescent anti-human C3 (Plate VII). In contrast, there was minimal fluorescence with fluorescein-labelled anti-human C4. The capacity of the tegument to bind C3 by alternate pathway activation was further demonstrated by experiments using human C2-deficient serum. With this C2-deficient serum there was a strong fluorescence with anti-human C3 but not with anti-human C4 (Table VIII).

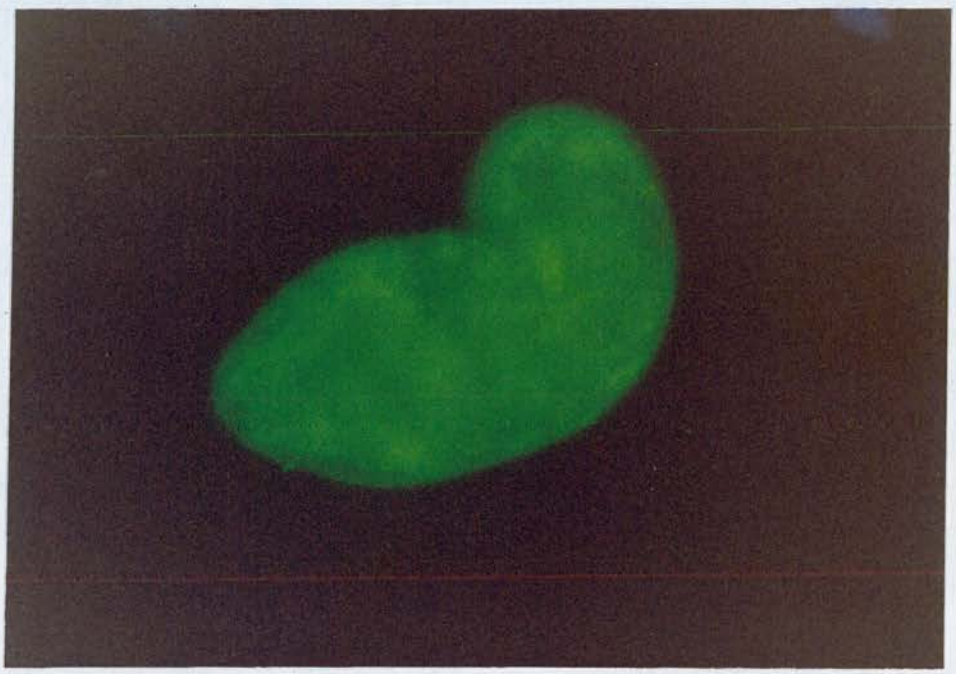

Plate IX

Fluorescence of schistosomula (incubated with anti-schistosomiasis serum and then with fresh normal human serum) in the presence of fluorescent labelled anti-human C3.

(x 400 magnification)

TABLE VIII

Fluorescence of schistosomula treated with antibody and/or complement in the presence of fluorescent-labelled anti-human C3 and anti-human C4.

The scoring of fluorescence was from 0 to ++++.

N.D. = not done.

	ANTI-C4	ANTI-C3
Normal serum (C)	(+)	+++
C2-deficient serum	0	+++
Normal serum + anti-schistosomula serum^{H} (Ab)	++	++++
Anti-schistosomula serum^{H} + C1, C4, C2, C3	N.D.	+++
Anti-schistosomula serum^{H} (Ab)	0	(+)
Normal serum^{H}	0	(+)
Medium alone	0	0

TABLE VIII

3.0 EFFECT OF VARIATION OF EFFECTOR CELL:TARGET RATIO

Experiments were performed to determine the optimum effector cell:target ratio needed for schistosomula killing in all three experimental systems using both granulocytes and mononuclear cells. In all three systems the percentage killing increased initially with the increase in the number of leucocytes per schistosomulum (Figs. 27, 28). However, like other killer cell systems previously described, the degree of killing of the schistosomula seemed to plateau and remained constant thereafter. In some experiments (not shown) the ratio was further increased up to 10,000 granulocytes or mononuclear cells per schistosomulum but the increase in killing was less than 10% than that achieved with 5,000 leucocytes per schistosomulum.

In all the experiments described in this section a ratio of 4,000 leucocytes per schistosomulum was used so as to avoid the difficulty which arises if more cells are used, e.g. clumping which renders counting of dead schistosomulum with adherent leucocytes very difficult.

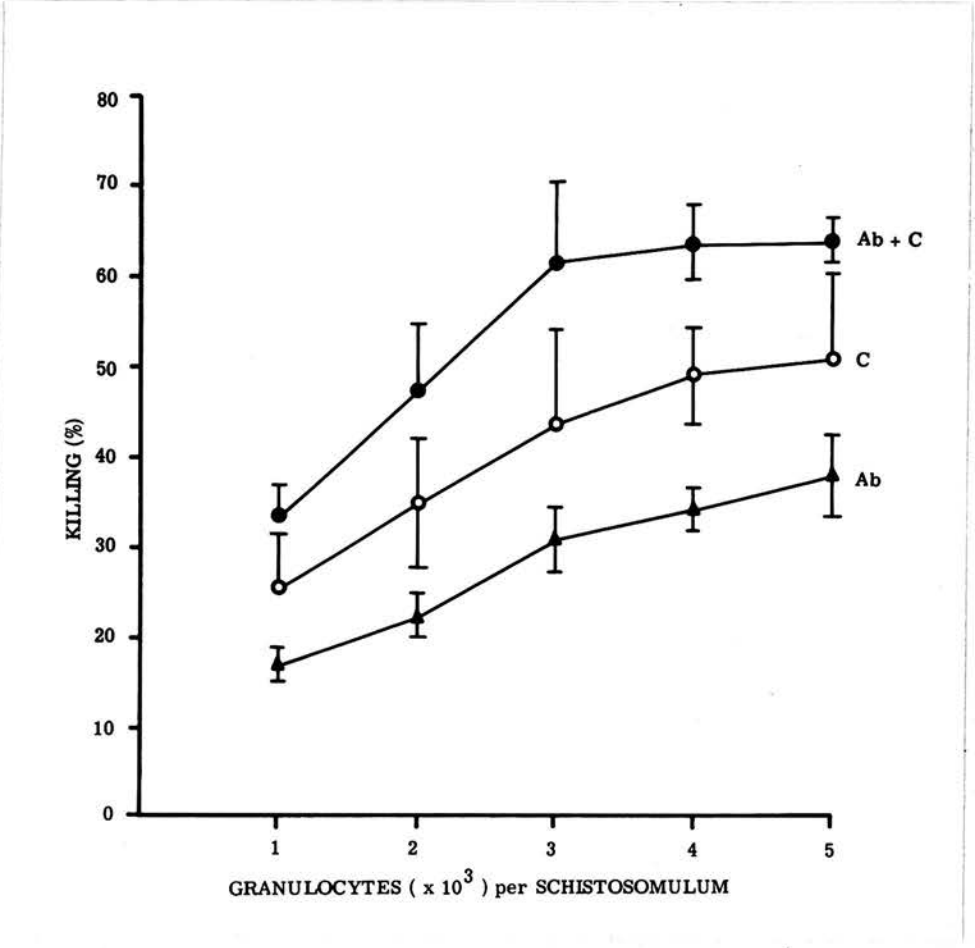

Fig. 27

The effect of varying the ratio of granulocytes: schistosomula on the killing of schistosomula coated with antibody (Ab) and/or complement (C).

The points represent the mean (± 1 S.D.) of five experiments.

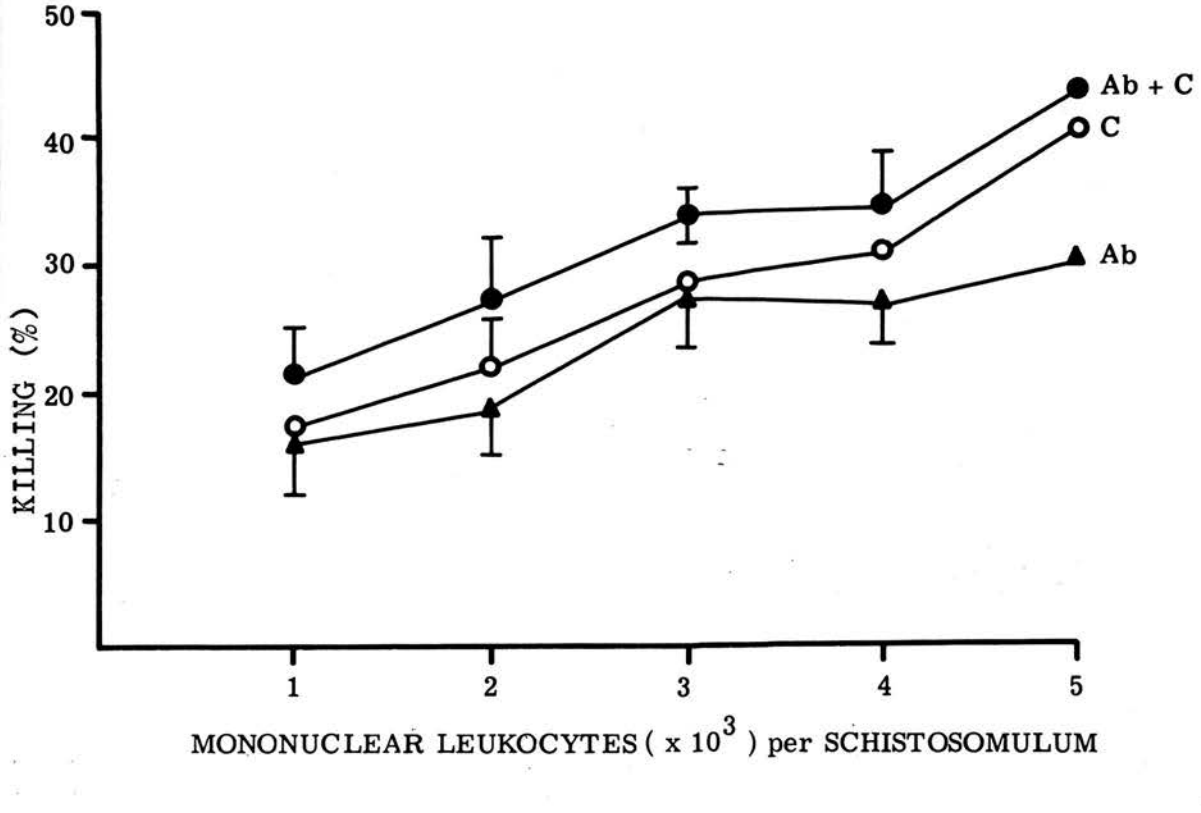

Fig. 28

The effect of varying the ratio of mononuclear cells: schistosomula on the killing of schistosomula coated with antibody (Ab) and/or complement (C).

The points represent the mean (± 1 S.D.) of four experiments (only one experiment was performed for 5 x 10^3 mononuclear leucocytes/schistosomulum).

4.0 PREFERENTIAL DAMAGE OF SCHISTOSOMULA BY EOSINOPHILS

Cell suspensions containing only eosinophils and neutrophils were prepared from the peripheral blood from each of seven healthy human volunteers. The percentage of eosinophils in these granulocyte suspensions was varied from approximately 3-63% and their capacity to kill schistosomula in the three experimental systems ('Ab alone', 'C alone' and 'Ab + C') was examined (Fig. 29). With 'Ab alone' the percentage killing ranged between 26 and 36% and was not affected by varying the ratio of eosinophils to neutrophils suggesting that the two cell types were equally effective in mediating schistosomula damage in this system. In contrast, with 'C alone' and 'Ab + C', an increase in the percentage of eosinophils was associated with a concomitant increase in the percentage of schistosomula killing which was highly statistically significant for both systems ($p < 0.001$) suggesting that preferential killing of schistosomula by eosinophils can only occur in the presence of complement. The increase in the percentage killing of schistosomula by increasing the eosinophil percentage was observed up to approximately 30% eosinophils, after which the effect appeared to remain relatively unchanged. However, the correlation coefficients were calculated for all the experimental points, i.e. including those points beyond which the effect appeared to plateau. With 'C alone', schistosomula killing for the seven experiments shown in Fig. 29 was in the range of 27 to 40% with neutrophil-rich (approximately 97%) suspensions to between 45 and 68% with suspensions rich in eosinophils (approximately 60%).

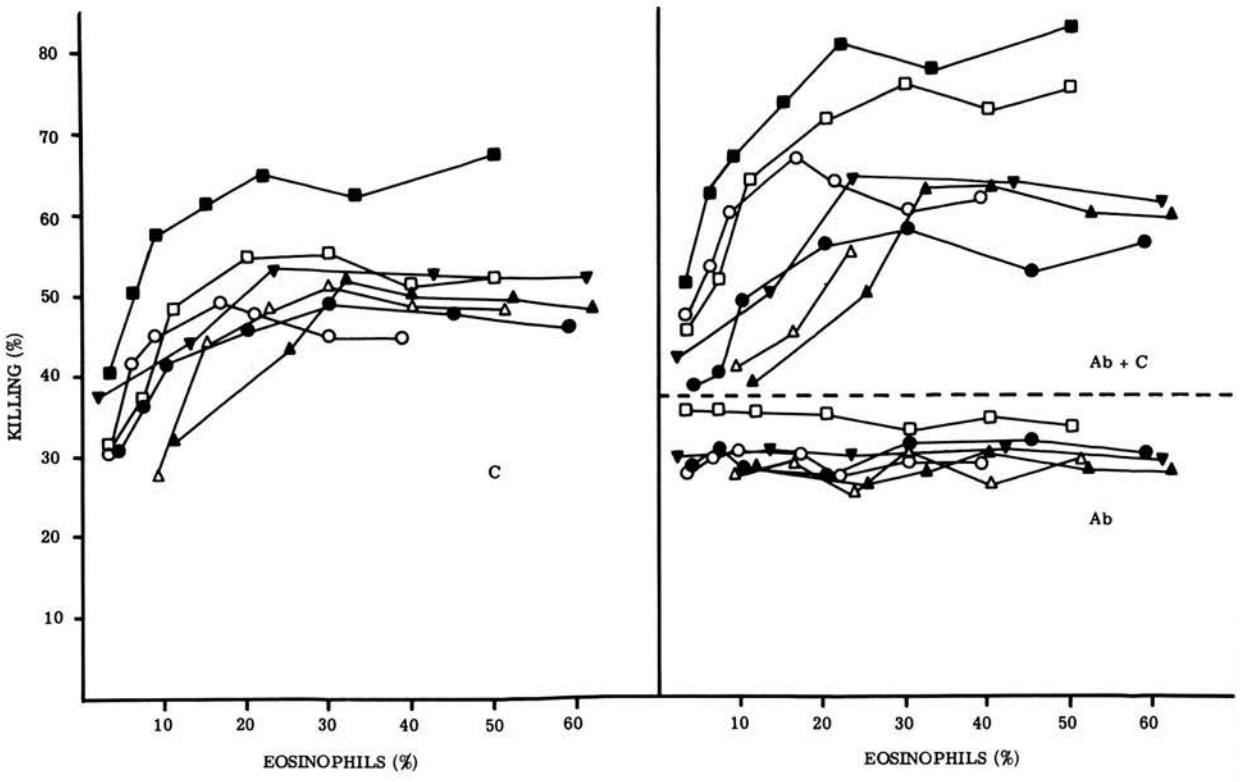

Fig. 29

The effect of increasing the percentage of eosinophils in the granulocyte suspensions on schistosomula killing using 'Ab alone', 'C alone' of 'Ab + C'.

Each symbol represents the results obtained from cells from the same donor studied on the same occasion in each experimental system. In two experiments only two systems were studied.

Correlation coefficients were calculated between the percentage killing and the eosinophil percentage in the donor granulocyte suspensions. The mean of the r values obtained in each system were tested for significance against zero using the Student t-test. The results were as follows:
(i) 'Ab alone' - not significant; (ii) 'C alone' - $p < 0.001$;
(iii) 'Ab + C' - $p < 0.001$.

Similarly, with the $^{s}Ab + C^{s}$ system killing ranged from between 37 and 50% to 53 and 82% with the neutrophil-rich and eosinophil-rich suspensions respectively.

Therefore, it was concluded that both neutrophils and eosinophils mediate schistosomula damage when complement is present, either alone or with antibody, but an increase in damage is demonstrable by increasing the percentage of eosinophils in the effector cell population.

5.0 SUMMARY

1. Using an *in vitro* assay system the capacity of human eosinophils, neutrophils and mononuclear cells to damage the larval stage of *Schistosoma mansoni* (schistosomulum) was studied. Schistosomula and leucocytes were incubated either with (i) 'antibody alone' (as serum from patients with schistosomiasis or its IgG fraction or as baboon anti-schistosomiasis serum), (ii) 'complement alone' or (iii) the combination of antibody and complement.

2. The mean efficiency of killing of schistosomula by granulocytes, i.e. mixtures of eosinophils and neutrophils, was 31% for 'Ab alone', 52% for 'C alone' and 70% for 'Ab + C'; controls gave 14% killing. Equivalent numbers of mononuclear leucocytes gave 22%, 31% and 37% killing with 'Ab alone', 'C alone' and 'Ab + C' respectively.

3. In all three experimental systems damage of schistosomula by granulocytes or mononuclear leucocytes was dependent on the ratio of effector cells to schistosomula. Furthermore, killing of schistosomula was directly related to the concentration of the antibody with 'Ab alone' and to the dilution of the complement with 'C alone' and 'Ab + C' systems.

4. Granulocyte dependent damage of schistosomula was directly related to the input of human C3 when the components of the classical pathway were added sequentially to schistosomula sensitized with antibody.

5. Increasing the eosinophil percentage in the granulocyte cell suspension produced no increase in schistosomula damage in the 'antibody alone' system,

suggesting that under these conditions eosinophils and neutrophils are equally effective in mediating schistosomula damage. In contrast, with 'C alone' and 'Ab + C' an increase in the percentage of eosinophils was associated with a concomitant increase in the degree of schistosomula damage which was highly statistically significant ($p < 0.001$).

6. From these studies it was concluded that under the appropriate experimental conditions:

(a) damage to schistosomula <u>in vitro</u> can be mediated by human eosinophils, neutrophils or mononuclear leucocytes in the presence of either 'Ab alone', 'C alone' or 'Ab + C'.

(b) the efficiencies of the three experimental systems were Ab + C > C > Ab irrespective of whether the effector cells were granulocytes or mononuclear leucocytes.

(c) preferential killing of schistosomula by human eosinophils, as compared to the neutrophils, can be demonstrated only when complement is present in the system either alone or in combination with antibody.

CHAPTER V - GENERAL DISCUSSION

CHAPTER V - CONTENTS

1.0 RECEPTORS FOR IgG AND COMPLEMENT ON HUMAN EOSINOPHILS AND NEUTROPHILS

Using both the rosette technique and immunofluorescence the presence of receptors for rabbit and human IgG on human eosinophils and neutrophils was demonstrated. The use of the rosette technique for the investigation of various cell membrane markers is well established. In the author's opinion this technique is easy to perform and relatively inexpensive. The main practical difficulties were in the preparation of the fixed slides and the counting procedure. It was found that the most convenient method for the purpose of this study was to smear the rosette suspensions on clean glass slides using a Pasteur pipette allowing the cell suspension to be drawn by surface tension. The slides were then dried quickly in air, using either a fan or hair dryer, fixed in methanol and stained with May Grunwald/Giemsa before they were counted. The use of wet preparation to count the rosettes was unsatisfactory because of the considerable difficulty in differentiating eosinophils rosettes from neutrophil rosettes, even when phase-contrast microscopy was used. Again the fixation and staining of the rosette slides had the advantage of making the preparations semi-permanent. This allowed the processing of large numbers of slides which can be stored for a short time and counted when convenient. In some early experiments cytocentrifuge preparations were made but the results were inconsistent, at least in the author's hands, possibly due to the added shearing force of the cytocentrifuge, although the rosettes prepared by this method appeared more impressive.

In counting the rosette slides care must be taken in selecting suitable fields, particularly with respect to the ratio and distribution of the indicator sheep red cells to the leucocytes. Only leucocytes with three or more adherent indicator red cells were counted as rosettes. In each slide 200 cells were counted and the numbers of rosettes were expressed as a percentage of the total number of cells counted.

In one series of experiments (Fig. 5) a direct immunofluorescent technique was used to demonstrate the presence of a receptor for human IgG on human eosinophils and neutrophils. Although it is known that the eosinophil can show non-specific granular autofluorescence (Fuerst et al, 1965) it was not very difficult to obtain satisfactory results by using this technique. However, it should be mentioned that counting of these fluorescing granulocytes was quite laborious for a method to be recommended for routine use.

Sheep red cells sensitized with rabbit IgG (EA_G^{rab}) formed rosettes with both human eosinophils and neutrophils in a dose-dependent fashion using increasing concentrations of rabbit IgG (Fig. 3). The percentage of neutrophil rosettes was approximately two and one half times that of eosinophil rosettes. These results are in agreement with the previous findings of Tai and Spry (1976) who demonstrated the presence of receptors for rabbit IgG on eosinophils from patients with eosinophilia. Messner and Jelinek (1970) previously described the presence of receptors for human IgG on human neutrophils using both an in vitro bactericidal-phagocytic assay and a rosette technique.

In the present studies experiments were performed to determine the optimum experimental conditions for detection of IgG receptors on both neutrophils and eosinophils. These receptors were better detected at 0°C rather than at 37°C. These results confirmed the previous findings of Wong and Wilson (1975) who were able to show better demonstration of IgG rosettes on human neutrophils at 4°C rather than at 37°C. They have suggested that it is possible that there might be some shedding of the IgG receptors at higher temperatures. However, there was no evidence for shedding of receptors at 37°C in the present study and the most likely reason for less IgG rosettes at 37°C is the marked degree of phagocytosis observed at this temperature.

Heat aggregated immunoglobulins possess many of the properties of antigen-antibody complexes and they appear to bind to the same membrane receptor sites on various haemopoietic cells (Dickler, 1974; Dickler and Sachs, 1974; Basten et al, 1975). Therefore, heat aggregated IgG was used previously by Dickler and Kunkel (1972) to demonstrate the presence of IgG receptors on human lymphocytes. Others have also used it to demonstrate the presence of a similar receptor on human granulocytes (Gupta et al, 1976). Furthermore, Hallberg (1974) showed that heat aggregated human IgG could inhibit the in vitro cytotoxic activity of human lymphocytes for chicken red cells sensitized with rabbit antibody.

In the experiments described earlier (Chapter IV, Section I) heat aggregated human IgG was used to detect

directly the presence of receptors for human IgG (Fig. 5) or to inhibit the rosette formation by human eosinophils and neutrophils with sheep red cells sensitized with rabbit IgG (Fig. 4). The results of these studies taken together with the results of the experiments in which sheep red cells sensitized with rabbit IgG (Fig. 3) formed rosettes with eosinophils and neutrophils, provided strong evidence for the presence of receptors for human and rabbit IgG on human eosinophils and neutrophils. The binding between rabbit or human IgG to the cell membrane of eosinophils and neutrophils is probably through the Fc region of the IgG, although during the present work no specific studies were undertaken to confirm this possibility. Again the sub-classes of IgG involved in the binding were not determined, although Tai and Spry (1976) were able to show that this receptor on human granulocytes binds to the Fc region of human IgG_1 and IgG_3. The demonstration of IgG receptors on human eosinophils is in agreement with the finding of Butterworth et al (1975) that the human eosinophil is the principal effector cell which mediates antibody-dependent killing of schistosomula of Schistosoma mansoni in vitro. The antibody in this system was identified as IgG (Butterworth et al, 1977b) and the adherence and killing of eosinophils to IgG-sensitized schistosomula may be dependent on the presence of freely available immunoglobulin receptors.

In the present study rosette formation was consistently detected between EA_G^{rab} and eosinophils both from healthy individuals and from patients with eosinophilia (Fig. 7). These findings were at variance with the report of Tai and

Spry (1976) who were unable to demonstrate an appreciable binding of EA_G^{rab} with human eosinophils from healthy donors. It is unlikely that this discrepancy was due to the use of ox red cells by these workers instead of sheep red cells which were used in the present study. Differences in the preparation of the EA_G^{rab} and the time of incubation allowed for the rosette formation may provide an explanation for these differences. Tai and Spry (1976) reported on six normal individuals using only a four-minute incubation time whereas in the present study eosinophils from 15 normal donors were studied using an incubation time of thirty minutes. In this respect it is also relevant that more recently the same workers have demonstrated that eosinophils from normal donors form EA_G^{rab} rosettes following overnight culture (Tai and Spry, 1978, personal communication).

Human eosinophils and neutrophils were tested for their rosette forming capacity with human group O Rh +ve red cells (R_1R_1 and R_2R_2) sensitized with anti-D antibodies. Neither the eosinophils nor the neutrophils formed rosettes with these sensitized human red cells. In contrast, the monocytes tested at the same time formed rosettes with the same indicator human red cells.

The use of Rh +ve red cells sensitized with anti-D antibody was reported previously by various workers as an established technique for the demonstration of the presence of receptors for human IgG on the human monocyte/macrophage series (Lo Buglio et al, 1967; Cline and Lehrer, 1968; Huber et al, 1969). Later Messner and Jelinek (1970) used the same method in an attempt to detect IgG receptors on

human neutrophils. However, with red cells sensitized with normal anti-D antibody there was no rosette formation by the neutrophil. In contrast, when they used Ripley anti-D antibody sensitized human red cells, 35% neutrophils formed rosettes as compared to 48% of the monocyte population. It is known that the Ripley anti-D differs in several ways from other conventional anti-D antibodies. For instance, it reacts with most human rheumatoid factors (Weller and Vaughan, 1956) and it is capable of fixing complement (Harboe et al, 1963). It should be noted, however, that more recently Gupta et al (1976) reported that they were unable to detect rosette formation between human eosinophils and human red cells even when these were sensitized with Ripley anti-D antibody. These findings are in agreement with the results described in this study where human red cells sensitized with anti-D antibodies did not form rosettes with either human eosinophils or neutrophils, although no attempt was made to sensitize the red cells with Ripley anti-D.

In another series of experiments (Fig. 6) in which an attempt was made to increase the amount of antibody uptake, human Rh +ve red cells were treated with the proteolytic enzyme, papain, before sensitization with anti-D antibodies and testing for rosette formation with eosinophils and neutrophils. However, these papainized red cells did not form rosettes with either eosinophils or neutrophils while rosette formation could be demonstrated with monocytes (Fig. 6). The papainization of red cells is a well documented method in red cell serology to enhance antibody/red cell interaction (Mollison, 1972) especially with antibodies of the Rhesus blood group. It is thought that the effect of papain

treatment on the red cell membrane is to increase the amount of antibody uptake (Masouredis, 1962; Hughes-Jones et al, 1964). It is also possible that the removal of the surface glycoprotein allows better contact with the antibody molecule. The rhesus antigen is thought to be part of the lipoprotein membrane of the red cell (Green, 1972) 'buried' between the glycoprotein chains bearing other red cell antigens such as A, B, etc. and this is thought to be one of the reasons why rhesus antibody molecules do not normally fix complement although they are of the appropriate IgG subclass (Mollison, 1972). It has also been reported that the action of papain on the red cell membrane may lead to the movement of the antigen sites in the membrane which is in a semi-fluid state (Voak et al, 1974; Romano et al, 1975) and thus the antibody molecules may form 'clusters' allowing a greater density of activated Fc receptors to be available. Papainization of Rh +ve red cells was also used to enhance the K-cell lysis of human red cells by human monocytes (Holm, 1972) and lymphocytes (Urbaniak, 1978).

Sheep red cells sensitized with rabbit IgM were also tested for their capacity to form rosettes with human eosinophils and neutrophils at both 0°C and 37°C (Fig. 8). Under the experimental conditions described there was no significant rosette formation by either the eosinophil or neutrophil with EA_M^{rab} and it was, therefore, decided to use these EA_M^{rab} cells in the preparation of complement coated intermediates to test for rosette formation by various complement components.

The presence of a receptor for IgM on certain subpopulations of human T and B lymphocytes had been reported over the last few years (Moretta et al, 1975; McConnell

and Hurd, 1976; Gmelig-Heyling et al, 1976). These observations were confirmed by Ferrarini et al (1976) who showed this receptor to be specific for the Fc portion of IgM. Later Pichler and Knapp (1977) described the presence of a similar receptor for IgM on chronic leukaemic cells. Also the presence of a similar receptor on normal human B lymphocytes after overnight culture was demonstrated (Ferrarini et al, 1977).

In early experiments performed in the present study neither the neutrophils nor the eosinophils formed rosettes with untreated sheep red cells although occasional rosette formation between some human polymorphonuclear cells and untreated sheep red cells has been reported previously (Hsu and Fell, 1974). Furthermore, Sher and Glover (1976) reported that human eosinophils can form rosettes with untreated sheep red cells in a similar manner to T lymphocytes. In view of these observations it was decided that this non-specific binding phenomenon should be closely evaluated. The rosettes formed between T lymphocytes and untreated sheep red cells are known to be unstable and are easily broken with various handling procedures. Several workers used a variety of test modifications to enhance the strength of binding between T lymphocytes and sheep red cells. Weiner et al (1973) reported that neuraminidase treatment of sheep red cells could enhance rosetting formation. Also Bentwich et al (1973) could demonstrate enhanced binding of sheep red cells to T lymphocytes in the presence of AB serum. The use of both dextran (Brown et al, 1975) and papainized sheep red cells (Wilson et al, 1975)

was also reported. In the present study the observation of Kaplan and Clark (1974), where the treatment of sheep red cells with the sulphhydryl reagent, AET, was shown to enhance the non-specific binding between these cells to human T lymphocytes, was utilized to evaluate the binding between human eosinophils and neutrophils to AET-treated sheep red cells. No rosette formation could be demonstrated with granulocytes, whereas lymphocytes tested at the same time consistently formed rosettes with the AET-treated red cells (Fig. 9).

In the present study no experiments were performed to test for the presence of surface immunoglobulin on human eosinophils and neutrophils. Gupta et al (1976) were unable to demonstrate the presence of surface immunoglobulin on human eosinophils.

Experiments using the rosette technique were performed to detect the presence of membrane receptors for various human complement components on human eosinophils and neutrophils. EAC14 and EAC3 formed rosettes with both eosinophils and neutrophils (Table V and Figs. 10, 11, 12, 13). Increasing amounts of these components, i.e. C4 and C3, were associated with a dose-dependent increase in rosette formation and with lysis on addition of the late components, suggesting that the percentage of eosinophil rosettes was directly proportional to the input of C4 and C3 on the indicator sheep red cells (Figs. 14, 15). It should be emphasized that the amount of C4 used to prepare the EAC3 intermediates was limited (400 effective molecules) so that the corresponding EAC14 gave the same percentage of

rosettes as the untreated red cells. Therefore, the neutrophil and eosinophil rosettes observed with EAC3 intermediates were not due to C4 and thought to be entirely C3-dependent. These results are in agreement with the findings of Gupta et al (1976) who were also able to demonstrate the presence of these receptors on human eosinophils and neutrophils. In the present studies, no experiments were undertaken to study the relationship between C4 and C3 receptors on human granulocytes. However, Gupta et al (1976) showed that EAC14 and EAC3b were both bound by the same eosinophil complement receptor, since preincubation of eosinophils in either fluid phase C4 or fluid phase C3c inhibited rosette formation with both types of intermediates. From similar specificity studies Ross and Polley (1975) earlier showed that C3b and C4 were bound by the same receptor on human lymphocytes.

Previously the inability of EAC3d cells to form rosettes with human neutrophils was reported (Ross et al, 1973; Eden et al, 1973). However, in the present study EAC3d cells, prepared by treating EAC3b cells with human C3b inactivator, formed rosettes with both eosinophils and neutrophils. These EAC3d cells, unlike the EAC3b cells, were immune adherence negative, resistant to lysis by the addition of C5-C9 and gave a stronger agglutination with monospecific anti-C3d than did the EAC3b cells, but they were not tested for rosette formation with Daudi lymphoid cells, which carry only receptors for C3d (Dierich et al, 1974b; Theofilopoulos et al, 1974). It should be emphasized here that in the present study, the term 'C3d

receptors' refers only to those receptors which will react with the C3b inactivator-treated EAC3b which are no longer immune adherence positive and which cannot be lysed by the addition of C5-C9. It is not intended to include the various complex molecular mechanisms which occur as a result of the action of the C3b inactivator on the C3b molecule (Harrison and Lachmann, 1978). For this reason more recent reports have referred to CR_2 (complement receptor type 2) instead of C3d receptors (Ross et al, 1978). The differences between the results presented here and those of earlier reports (Ross et al, 1973; Eden et al, 1973) may be explained by differences in technology. Indeed, more recently Ross et al (1978) have also demonstrated the presence of receptors for C3d receptors (CR_2) on human neutrophils. They have taken advantage of the finding that the neutrophil cell density increases with maturation and so by using density gradient centrifugation they were able to separate various fractions of neutrophils. These different neutrophil fractions were thought to represent different individual cell maturation stages. The presence of C3d receptors was found to be associated with the less mature cells and it was suggested that this receptor may be lost after full cellular maturation.

Rosette formation by neutrophils and eosinophils with EAC14 was inhibited by functionally pure human C2 which was free of C3b inactivator (Table I). Although it was previously reported that C2 did not inhibit EAC14 immune adherence (Cooper, 1975), it should be mentioned that in the present study the inhibition of EAC14 rosette formation

was performed at 0°C to minimize C2 decay. By allowing C2 to decay at a higher temperature, rosette formation by EAC14 could be restored (Tables I, II). These findings suggest that C4-haemolytic and granulocyte-binding sites are likely to be closely related.

The functional significance of C4 and C3b receptors on various phagocytes may be difficult to explain in relation to the presence of C3b inactivator that renders EAC14b and EAC3b complexes non-reactive with the immune adherence receptors. Antigen-antibody complexes in plasma fix C4b and C3b which are rapidly cleaved by plasma C3b inactivator into the respective c and d fragments (Cooper, 1975; Ruddy and Austen, 1971). The C4b cleavage products, C4c and C4d, are believed to be non-reactive with complement receptors (Bokisch and Sobel, 1974), while the complex-bound C3b cleavage product, C3d, only has activity with C3d receptors. In vivo, however, C4b sites on antigen-antibody complexes may be partially protected from C3b inactivator action by formation either of a short-lived complex with C2 (Cooper, 1975) or with the low molecular weight C4b binding protein (Nagasawa and Stroud, 1978). This may help to preserve C4b sites long enough to allow binding of antigen-antibody complexes to the immune adherence (C4 and C3b) receptor-bearing phagocyte. There is also a similar complexity in the in vivo reactions involving bound C3b. It is known that when the antigen-antibody complexes are sheep red cells (EAC), the cell-bound C3b cleavage product, C3d, has little opsonic activity (Gigli and Nelson, 1968; Stossel et al, 1975; Ehlenberger and Nussenzweig, 1977). However, under normal circumstances in vivo C3-coated

bacteria and yeast are usually phagocytosed whilst erythrocyte target cells are likely to be handled instead by lysis with natural antibody and complement. It was shown also that yeast cell wall-derived zymosan surface membranes differ from sheep red cell membranes in that alternate pathway-derived C3b is bound in such a way that it is relatively resistant to C3b inactivator cleavage (Fearon and Austen, 1977). Therefore, it seems that with both yeast and bacteria C3b can act as an effective opsonin despite the presence of C3b inactivator.

The relationship between granulocyte complement receptors and eosinophilia was studied. There was a significant decrease in the percentage of eosinophil rosettes with EAC14 and EAC3b in patients with eosinophilia (Fig. 7). This may possibly be due to the presence of smaller numbers of complement receptors on immature eosinophils which appear more commonly in the peripheral blood of these patients as a result of the stimulation of their production and release from the bone marrow. This concept is supported by the work of Gupta (1977), who studied the sequence of appearance of surface receptors for complement and immunoglobulin on human lymphocytes, and showed that the complement receptors were the last to appear during lymphocyte maturation. In addition, Rabellino and Metcalf (1975) were unable to demonstrate receptors for C3 on mouse eosinophil colony cells grown in vitro, whereas in the same study IgG receptors were demonstrated. This is in agreement with the present study in which there was no difference between eosinophils

rosetting with EA_G^{rab} in patients with eosinophilia when compared to controls.

There was no apparent association between the percentage of eosinophil or neutrophil rosettes with EAC14 or EAC3b and the degree of eosinophilia. Also there was no association between the percentage of EAC14 and EAC3b rosette formation and the disease states.

These results described in the present study are in agreement with the previously described surface receptors on human monocytes (Huber and Fudenberg, 1968; Huber et al, 1968) in that, like monocytes, human granulocytes bear receptors for IgG, C4, C3b and C3d. This suggests that there are similarities in terms of recognition of opsonized particles by all circulating phagocytic cells.

2.0 ENHANCEMENT OF HUMAN EOSINOPHIL COMPLEMENT RECEPTORS BY PHARMACOLOGICAL MEDIATORS

Using the rosette technique it was shown that the expression of the human eosinophil complement receptors can be enhanced by agents previously recognised to be selectively chemotactic for human eosinophils. The actual mechanisms involved in the increased receptor expression are as yet unclear. The elucidation of such mechanisms is made more difficult by the fact that little is known about the physiochemical characteristics or the arrangement of the complement receptors on the cell membrane.

It should be mentioned that during this chapter the various eosinophilotactic agents, i.e. the ECF-A tetrapeptides, histamine and imidazoleacetic acid, were consistently referred to as being chemotactic only in the sense that they cause the eosinophil to migrate *in vitro* towards a gradient across a micropore filter. Further studies are needed where all these agents are examined, either alone or in combination, using the 'chequerboard' titration method so as to distinguish true chemotaxis from chemokinesis (Zigmond and Hirsch, 1973; Keller *et al*, 1977). Also, the relationship between chemotaxis, chemokinesis and complement receptor enhancement may need to be defined.

The ECF-A peptides and histamine were apparently unable to enhance neutrophil or monocyte complement receptors. This demonstrates further the more intimate relationship between these anaphylaxis-related agents and the eosinophil leucocyte. It was not possible for the effect of the ECF-A peptides and histamine on neutrophils

and monocytes to be revealed when the amount of C3 input on the indicator red cells was reduced, since in experiments where the C3 input was adjusted to give less than half the number of rosetting cells (i.e. 1000 effective molecules) there was no increase in the percentage of the rosetting neutrophils or monocytes following the incubation with either the peptides or histamine (Table VI).

The ECF-A peptides, histamine and imidazoleacetic acid had no effect on the number of EA_G^{rab} rosettes formed with any of the leucocytes tested. The apparent inability of these agents to enhance receptors for IgG on the eosinophil may have been due to the slight differences in the experimental conditions since rosette formation with IgG-coated red cells was performed at 0^oC whereas 37^oC was used as the optimal temperature for EAC rosette detection. On the other hand, C3d receptors (which were also identified at 37^oC) were unaltered by these same pharmacological agents. Therefore, the difference in the incubation conditions seems unlikely to explain the inability of the mediators to influence eosinophil IgG receptors. It should be stated that at 37^oC, in contrast to 0^oC, there was considerable phagocytosis of EA_G^{rab} by granulocytes and it was, therefore, impractical to perform the EA_G^{rab} receptor enhancement studies at a higher temperature. The use of more sensitive techniques, e.g. immunofluorescence, might be more useful in studying the possible effects of these agents on enhancement of EA_G^{rab} receptors.

In the studies described here optimal receptor enhancement was achieved during a relatively short incubation

period (60 min) which would make it rather unlikely that new receptors were generated during this time. The treatment of eosinophils by these agents may have unmasked previously hidden receptor sites or facilitated some form of externalization. For instance, complement receptors may behave like those for low density lipoprotein on human fibroblasts, which are believed to have a continuous ordered motion around and through the cell (Anderson et al, 1977). Another possibility is that the pharmacological agents may lead to 'unfolding' of the cell membrane to expose more receptor sites which would have otherwise remained inaccessible. Alternatively, complement receptors may be composed of subunits which, following treatment with agents that can cause cell migration, lead to their association with consequent binding of more indicator cells. Some of the possible mechanisms involved in receptor enhancement are represented diagrammatically in Fig. 30. One further explanation may be dependent, not on gross movements of the receptors or their subunits, but on an increase in the binding affinity or increased 'stickiness' of that part of the cell membrane which adheres to complement-bearing particles.

Although the use of the rosette method for studying cell surface markers is well established, a major disadvantage, which applies particularly to the present study, is the difficulty in measuring the degree of binding between the cell membrane and individual indicator erythrocytes. It was found that eosinophils treated with the ECF-A peptides, histamine or imidazoleacetic acid not only increased the percentage of cells bearing three or more

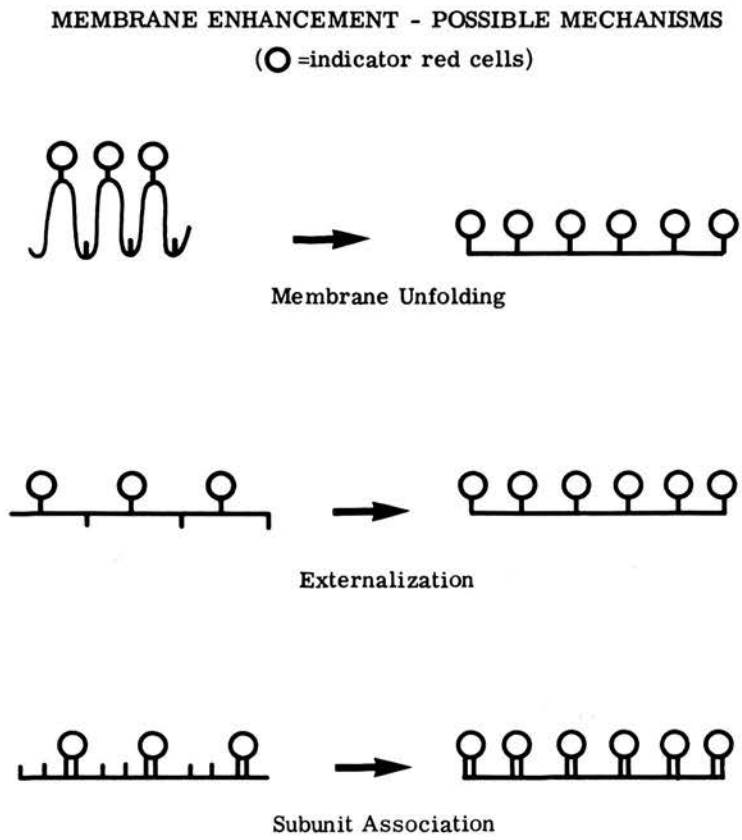

Fig. 30

Diagrammatic representation of some of the possible mechanisms of receptor enhancement.

red cells, but the red cell binding appeared far stronger than that observed in the untreated controls. From these observations it became evident that the rosette technique was not the ideal method and that more detailed studies in which other more sensitive techniques, which will facilitate measurements of the binding affinity, are required for the appreciation of the full extent of the receptor enhancement as a new biological phenomenon.

It was further shown that 'non-eosinophilotactic' mediators of anaphylaxis, e.g. bradykinin, prostaglandins (PGE_1, E_2 and $F_{2\alpha}$) and 5-hydroxytryptamine, and other agents closely related chemically to histamine, but chemotactically inactive such as histidine and the major catabolites of histamine (1,4-MeHm, N-AcHm and 1,4-MeImAA) when tested for C3b receptor enhancement on eosinophils, were without effect. This finding suggests that the enhancement of complement receptors is a chemotaxis-related phenomenon. It is now possible to say that the complement receptor enhancement may be a general biological phenomenon applicable to all those cells which both respond in chemotaxis and bear complement receptors, i.e. agents which promote cell migration can also enhance complement receptors. For example, the synthetic N-formylmethionyl peptide, F-Met-Leu-Phe, previously shown to be chemotactic for the neutrophil (Schiffman et al, 1975) enhanced neutrophil C3b receptors (Salter, D.M., unpublished observation). Similarly, agents having a recognised chemotactic activity for mononuclear phagocytes including casein (Keller and Sorkin, 1967) and 'lymphokines' (Snyderman et al, 1972)

(prepared from lymphocytes incubated with phytohaemagglutinin) enhanced C3b receptors on monocytes (Glass, E.J., unpublished observations).

Experiments performed to study the effect of various combinations of the ECF-A peptides and histamine on C3b receptor enhancement gave quite different results to similar studies previously reported for eosinophil chemotaxis (Turnbull et al, 1977). Whereas, with chemotaxis, mixing of the agents seemed to abrogate the response (Turnbull et al, 1977), with receptor enhancement there was neither abrogation, addition or synergism when various mixtures of the mediators were tested. It is possible that the previously reported mixing experiments (Turnbull et al, 1977) may have induced a form of chemotactic 'deactivation' under the experimental conditions used, a phenomenon which may not apply to receptor enhancement by the ECF-A tetrapeptides or histamine.

It was observed during the course of these receptor enhancement studies that there were two patterns of response to histamine in terms of its effect on eosinophil C3b receptors (not shown in the results). In one type of response, eosinophils showed marked receptor enhancement similar to the response of the ECF-A peptide, whereas in the other type histamine produced a much lower degree of enhancement. It was previously reported that there were alterations in various disease states in terms of the eosinophil chemotactic response to histamine and the ECF-A peptides (Bryant et al, 1977). In the present study no attempt has been made to relate the degree of receptor enhancement to various disease

states. However, an altered response in disease similar to that previously reported in relation to eosinophil chemotaxis may also apply to the receptor enhancement phenomenon.

The studies described here indicate that an important biological function of chemotactic agents may be their capacity to render leucocyte complement receptors more freely available, thereby increasing the magnitude of adhesion to opsonized particles. This raises the possibility that leucocytes are primed for adhesion process prior to their actual attachment. Furthermore, eosinophil complement receptor enhancement represents a previously unrecognised biological activity for the ECF-A tetrapeptides, histamine and imidazoleacetic acid. This activity may be more relevant to the role of eosinophils as cytotoxic cells against the larvae of certain helminths where the IgE-mediated release of chemical mediators is well recognised.

3.0 THE PARTICIPATION OF ANTIBODY AND/OR COMPLEMENT IN EOSINOPHIL-DEPENDENT KILLING OF SCHISTOSOMULA OF SCHISTOSOMA MANSONI

In the experiments described in section IV (see Results) a direct visual assay for helminth damage was used. In this assay, schistosomula killed by adherent leucocytes were easily recognised and quantitated by their distinctive appearance. The direct visual assay has the advantage of being relatively simple and inexpensive as compared, for instance, with the ^{51}chromium release method and was found, in the author's hands, to compare favourably with the latter in terms of sensitivity and reproducibility. However, one disadvantage is that it is rather laborious and time-consuming especially if many wells are to be counted. Previously in similar studies (Butterworth et al, 1974; Butterworth et al, 1975; Butterworth et al, 1977a, b) the ^{51}chromium release assay was used as the method for measuring damage of the schistosomula and the results were essentially similar to those presented here. Indeed, recently Mackenzie et al (1977) have shown that by using both methods, i.e. the direct visual assay and the ^{51}chromium release assay, the schistosomula damage proceeded in parallel.

In the present studies three experimental systems were used, i.e. (a) 'antibody alone', (b) 'complement alone', where fresh normal human serum was used as a source of complement through the activation of the alternate complement pathway by the tegument of the schistosomula, and (c) the combination of antibody and complement.

With the 'Ab alone' system the percentage killing of

schistosomula by human leucocytes found in this study was in agreement with studies of Butterworth et al (1975) and Butterworth et al (1977a, b) who found that killing (approximately two and one half times) was significantly greater than the appropriate control. These percentages were significantly higher than the control (Table VII). With 'complement alone' the results are also in agreement with a previous study in which schistosomula coated with rat C3 were shown to be more susceptible to damage by rat eosinophils than those coated with IgG (Ramalho-Pinto et al, 1978). However, in the present study using human leucocytes and complement, neutrophils and mononuclear cells were also studied in addition to the eosinophil. With all these cell types complement-coated schistosomula were more susceptible to killing than schistosomula coated with antibody alone. Unlike the previous study in the rat (Ramalho-Pinto et al, 1978) the degree of killing with 'C alone' in the present study was consistently less than that previously reported with the rat eosinophil and complement, i.e. 52.9% as compared to approximately 90%. This discrepancy may be due to the fact that in the previous study in the rat (Ramalho-Pinto et al, 1978) schistosomula were used only 3 hr after transformation from cercariae whereas in the present study they were used after approximately 24 hr. In this respect it is worth noting that Ramalho-Pinto et al (1978) showed previously that complement-dependent adherence in 5-day-old schistosomula was negative. Alternatively, the results may simply reflect the species difference between the two studies.

The combination of antibody and complement was studied since it might be more relevant to the *in vivo* situation in resistance to reinfection. This combination, i.e. $^{s}Ab + C^{s}$, was found to be the most efficient system with both cell types (granulocytes and mononuclear cells) in terms of their capacity to cause schistosomula damage. A similar system was previously described by Dean *et al* who found that schistosomula coated by rat (Dean *et al*, 1974) and guinea pig (Dean *et al*, 1975) antibody and complement were susceptible to damage by neutrophils. Under the conditions described in the present study schistosomula killing by $^{s}Ab + C^{s}$ in the absence of leucocytes was minimal (Table VII), which also confirmed the previous findings of Dean *et al* (1974).

With all three experimental systems used the mononuclear cells (which contained less than 3% granulocytes) were relatively less effective than the granulocyte suspensions in terms of their capacity to mediate schistosomula damage. In the present study no attempts were made to determine whether individual cell types which comprised the mononuclear leucocytes, i.e. subpopulations of lymphocytes or monocytes, were more or less effective on a cell to cell basis, when compared to eosinophils or neutrophils. Nevertheless, in this respect, the finding that mononuclear cells can mediate schistosomula damage is in agreement with the findings of Capron *et al* (1975) who described schistosomula killing by rat macrophages. However, these workers found that killing by macrophages was dependent on IgE antibody. In the experiments described in this study the participation

of IgE was virtually excluded since all the anti-schistosomula sera used as antibody source were heat-inactivated for 1 hr at 56°C. Furthermore, evidence was provided that the mediating antibody class was IgG.

In the 'Ab alone' system killing was related to the concentration of the anti-schistosomula serum and with 'C alone' and 'Ab + C' to the amount of complement (Fig. 24), although it might be argued that it is not valid to compare schistosomula coated with (1) whole undiluted fresh serum as a source of complement with (2) undiluted anti-schistosomula serum in terms of susceptibility to killing by various leucocytes. For instance, the affinity of the antibody in the 'Ab alone' system may not have been sufficient to allow full expression of leucocyte-mediated schistosomula damage. However, this was unlikely since the 17 sera tested from patients with schistosomiasis, all gave killing within a fairly narrow range (25-45%). Furthermore, the serum samples were obtained from patients at different stages of the natural history of the disease indicating that they probably represented a broad spectrum of the anti-schistosomula antibody response. These data, therefore, taken together with the previous findings in the rat (Ramalho-Pinto et al, 1978) suggest that complement-coated schistosomula are particularly susceptible to damage by various leucocytes.

During the course of the present study no attempts were made to study quantitatively the degree of leucocyte adherence to schistosomula in relation to killing. However, it was consistently observed that cell adherence to antibody-coated schistosomula could occur without appreciable

schistosomula damage. In contrast, with the 'C alone' and 'Ab + C' systems, adherence and damage were directly related. In a previous study, using the rat cells, where adherence and killing were observed in parallel it was found that antibody-mediated cell adherence can occur without appreciable schistosomula damage (Ramalho-Pinto et al, 1978). Furthermore, in a more recent ultrastructural study (McLaren et al, 1978) it was shown that adherence of rat eosinophils to schistosomula coated with complement resulted in an earlier and more severe damage than antibody-dependent destruction.

Experiments were performed to study the capacity of schistosomula to activate the human complement system by the alternate pathway. In these studies the main evidence was obtained in experiments using C2-deficient serum (Table VIII) although the possibility that normal human serum may contain small amounts of natural anti-schistosomula antibody was not fully excluded since with normal serum there was a minimal fluorescence with anti-C4 (Table VIII). Previously Ramalho-Pinto et al (1978) showed that schistosomula can activate the alternate pathway of the rat complement system. The activation of the alternate pathway of complement by cercarial glycoprotein has also been reported (Machado et al, 1975). It seems likely that, with schistosomula, surface glycoproteins are also involved in complement activation by the alternate pathway. With fluorescent-labelled anti-C3, immunofluorescence was observed to be evenly distributed over the entire surface of the schistosomulum (Plate IX) indicating that the activating principle is an integral part of the tegument.

Furthermore, experiments were performed where senstiized schistosomula were coated by the sequential addition of the purified human complement components C1, C4, C2 and C3. Using this approach it was shown that susceptibility to damage by granulocytes of the schistosomula coated with antibody could be augmented with the purified classical pathway components and to be dependent on the input of C3 (Fig. 26). The presence of C3 on these schistosomula was also detected by immunofluorescence (Table VIII). With the addition of the purified complement components, as well as with 'C alone' and 'Ab + C' systems, the adherence and damage were presumably mediated via C3b and the appropriate receptors on the eosinophils and neutrophils, although these cell types were also shown to bear receptors for C4 and C3d. At the present time the role of these receptors in mediating adherence and possibly damage is not known. However, it is unlikely that the adherence and killing in experiments using the purified components was mediated via C3d and its appropriate receptors since the C3b inactivator was not present in the reaction mixtures.

Experiments were performed to determine the antibody class mediating the damage in the 'Ab alone' and 'Ab + C' systems. This was achieved by using purified IgG, free from detectable IgM and IgA. There was very little difference between whole anti-schistosomula serum and the purified IgG fraction in terms of their capacity to mediate granulocyte-dependent killing in those two systems (Fig. 25). However, a serum fraction free of IgG and containing comparable

amounts of IgM and IgA to those contained in the whole serum had no killing effect. This finding confirmed previous observations by Butterworth et al (1977b) who found that antibody-dependent schistosomula damage was mediated by IgG.

Since it became evident that both neutrophils and eosinophils can mediate schistosomula damage in all three experimental systems, experiments were designed to investigate the relative efficiency of eosinophils and neutrophils in schistosomula killing in the three experimental systems. For these studies cells from healthy donors were used because it was previously reported that eosinophils from eosinophilic patients with schistosomiasis were relatively ineffective in antibody-dependent cytotoxicity (Butterworth et al, 1975). Preferential killing of schistosomula by one cell type can be demonstrated by testing a number of cell suspensions, prepared from the same donor, in which the percentage of this cell type was varied whilst keeping the total cell count constant. Experiments performed using this approach, with seven different donors, showed that increasing the percentage of the eosinophils in the cell suspension resulted in increased killing of schistosomula but only in systems containing complement either alone, or in combination with antibody (Fig. 29). However, with 'Ab alone' there was no alteration in the schistosomula killing following the increase in the eosinophil percentage and it appeared that both eosinophils and neutrophils were equally effective in mediating schistosomula killing. These findings, with 'Ab alone', differed from those of Butterworth et al (1975) and Butterworth et al (1977a) who reported that

eosinophils were the only human blood leucocytes capable of mediating appreciable antibody-dependent killing of schistosomula. These workers prepared schistosomula by cercarial penetration of skin whereas organisms transformed artificially by mechanical procedures were used here. However, this is unlikely to explain these differences since there is no evidence that schistosomula transformed artificially by mechanical separation of the tails differ from schistosomula recovered after cercarial penetration of isolated skin. Immunofluorescence studies indicated that both types of schistosomula could bind serum from mice immune to Schistosoma mansoni and both had a comparable infectivity when injected intravenously in mice (Brink et al, 1977). In the experiments described here a different assay was used from that used by Butterworth et al (1975), i.e. killing was measured by a direct visual assay, whereas Butterworth et al (1975) used ^{51}chromium release as a measure for schistosomula damage. It is also unlikely that these different assay systems may explain the discrepancies since using both methods schistosomula damage has been shown to proceed in parallel (Mackenzie et al, 1977). The supporting media used in the present study were RPMI-1640 and medium 199, in contrast to Eagle's minimal essential medium supplemented with 10% heat-inactivated foetal calf serum as used by Butterworth et al (1975). The most likely reason for the difference in the findings with respect to the 'Ab alone' system is the difference in the experimental design, rather than the difference in the culture conditions and assay. Butterworth et al (1977a) compared the degree of

schistosomula damage by eosinophil-rich suspensions from seven individuals and in six of these dose-response curves were obtained by varying the effector cell/target ratio. There are difficulties in demonstrating a specific property for the eosinophil, or any other cell type, using this approach since it assumes that preparations from different individuals containing the same percentage of the cells under study are equally effective. Because of variability of cells obtained from different donors in terms of biological efficiency, this assumption might not always be valid. Therefore, it is important to keep the effector cell/target ratio constant and to 'dose-response' by increasing the concentrations of eosinophils using several suspensions from the same donor, thereby largely overcoming the difficulties of variations between different individuals.

It was previously shown by Butterworth et al (1975) that the use of monospecific anti-eosinophil serum abrogated antibody-dependent leucocyte damage to schistosomula. In the present study no experiments were performed using monospecific anti-eosinophil serum.

Although preferential damage by eosinophils could be demonstrated in the two systems incorporating complement, it was evident that the neutrophil was also an effective cytotoxic cell (Fig. 29). For instance, with the highly neutrophil-rich suspensions (approximately 97%) killing with 'C alone' was between 27 and 40% and between 37 and 50% for 'Ab + C'. If schistosomula damage by the neutrophil-rich suspensions was entirely due to contamination by eosinophils then this would imply that, from the results shown in Fig. 29,

a ratio of 120-400 eosinophils per schistosomulum was sufficient to cause appreciable damage. This is extremely unlikely since it is known from the individual eosinophil counts of the granulocyte suspensions in experiments performed to determine the effector cell/target ratio used in the present studies (Fig. 27) that a ratio of 400:1 was insufficient to cause schistosomula damage which was greater than that caused by controls.

With 'C alone' and 'Ab + C' the effect of increasing the eosinophil percentage in the granulocyte preparations seemed to plateau between approximately 20 and 35% eosinophils (Fig. 29). The reasons for this 'plateauing' effect are unclear at the present time although this may be a reflection of the maximal degree of killing possible in this in vitro assay.

In the present studies no attempts were made to study the exact mechanisms of schistosomula damage by human leucocytes in the three experimental systems. Previously the reactions between human eosinophils and schistosomula in the 'Ab alone' system were examined by phase contrast and electron microscopy (Butterworth, A.E., personal communication). It was shown that the reaction was initially characterized by a tight adherence between the eosinophil and the schistosomulum, a feature which was not seen in control preparations. This stage was followed by eosinophil degranulation and by the appearance of an electron-dense deposit on the surface of the worm. Some eosinophils might then withdraw from the surface, while others might be seen in the process of phagocytosing

fragments of the extensively damaged schistosomula. While studying the ultrastructural changes occurring between rat eosinophils adhering to schistosomula in the presence of rat immune serum, McLaren et al (1977) showed that the initial response of the rat eosinophil was degranulation leading to the formation of large cytoplasmic vacuoles. Peroxidase was discharged into these vacuoles as a consequence of degranulation. Eventually the vacuoles became connected to the adherent basal membrane of the eosinophil, and peroxidase was directly secreted on to the surface of the worm. However, these workers were unable to detect any morphological evidence to suggest that these particular reactions following eosinophil adherence could affect the integrity of the schistosomulum surface. More recently, ultrastructural evidence for both complement and antibody-dependent damage to schistosomula by rat eosinophils was provided by McLaren et al (1978) who observed the appearance of focal lesions in the tegument of the schistosomula following the secretion of enzymes by the eosinophils on to the parasite surface. The cells were observed within these lesions and later between the basal plasma membrane of the tegument and the underlying interstitial material. It was, therefore, suggested that the eosinophils are responsible for prising the tegument away from the body of the worm and the detached tegument would show evidence for further degradation. It was also observed that the complement-dependent adherence of rat eosinophils resulted in earlier and more severe damage to schistosomula than when adherence occurs through IgG (Fc) receptors. In the same study, rat

eosinophils were shown to adhere to C3-coated Sepharose beads, but evidence of enzyme secretion was only obtained when the target was a schistosomulum.

It is possible that the adherence by human eosinophils to complement-coated schistosomula may lead to a series of events and membrane changes, similar to those described with the rat eosinophil, which will ultimately cause damage of the parasite. However, this is yet to be substantiated. In any case, it seems, from the results described in the present study with the human eosinophil together with those previously described in the rat eosinophil (McLaren et al, 1978), that eosinophil adherence to complement-coated schistosomula will generate a more effective mechanism whereby eosinophil-derived enzymes lethal for schistosomula are released.

4.0 CONCLUDING COMMENTS AND SUGGESTIONS FOR FUTURE STUDIES

The aims of the present studies were primarily to look for the presence of surface markers for immunoglobulins and complement on human eosinophils, to study the ways in which the expression of these receptors might be modulated, and finally to investigate the role that the human eosinophil might play via its membrane surface receptors in schistosomula killing. Eosinophils used in this study were usually obtained from patients with eosinophilia although in certain experiments cells from healthy controls were also studied. It was not possible on any occasion to obtain a 100% pure eosinophil preparation, and neutrophils were the usual contaminating cell. However, their unwanted presence was useful, in some ways, since it provided both control and comparative measurements.

The presence of receptors for IgG on both eosinophils and neutrophils was confirmed. No attempts were made to define the subclasses of IgG involved. Also the presence of receptors for various human complement components were studied. Human eosinophils and neutrophils were shown to bear receptors for C4, C3b and C3d. The presence of C4 and C3b receptors was previously reported by Gupta et al (1976). The inability of human neutrophils to form rosettes with EAC3d cells was reported earlier (Ross et al, 1973; Eden et al, 1973). However, in a recent report Ross et al (1978) have demonstrated the presence of C3d receptors on less mature neutrophils and they claimed that this receptor is lost during full cellular maturation.

The results presented in this study are consistent with

the previous findings on surface receptors on human monocytes (Huber and Fudenberg, 1968; Huber et al, 1968) in that, like other phagocytes, the eosinophils and neutrophils bear receptors for C4, C3b and C3d. Phagocytosis by the eosinophils is considered to be relatively less important than that by monocytes or neutrophils and the relative paucity of the eosinophil receptors may explain its poor phagocytic response. Eosinophils are now believed to be involved in certain major processes in the body, i.e. they may act as a cytotoxic-killer cell against certain helminths and they may have a homeostatic effect in immediate hypersensitivity reactions. Both these roles presumably require an effective recognition mechanism enabling contact between eosinophils and target cells which in turn may depend on immunoglobulin and/or complement receptors.

The ECF-A tetrapeptides, histamine and imidazoleacetic acid were shown to enhance the expression of complement (C4 and C3b) receptors on human eosinophils. This finding represents a previously unrecognised important biological activity for mediators of anaphylaxis known previously to be selectively chemotactic for eosinophils. Furthermore, the role of other chemotactic factors (casein and lymphokines; and F-Met-Leu-Phe) in mediating complement receptor enhancement on monocytes and neutrophils was demonstrated (Glass, E.J. and Salter, D.M., unpublished observation). These observations on the three cell types indicate that complement receptor enhancement by chemotactic factors may be an important general biological phenomenon related to all cells which respond in chemotaxis and bear complement receptors.

The exact way in which these chemotactic agents may affect the cell membrane receptors is unclear at the present time although various possible mechanisms are suggested (Fig. 30). However, more detailed studies are needed in which the whole phenomenon of complement receptor enhancement is studied at the membrane molecular level. Such studies may clarify the way in which chemotactic agents trigger the cell membrane to respond by receptor enhancement. Such elucidation will possibly be made more difficult by the fact that both the physiochemical nature and the arrangements of the complement receptors on the cell membrane are far from being clear at the present time.

In all the studies described in this thesis in relation to complement receptor enhancement no attempts were made to study the relevance of the enhancement, as a phenomenon, to various other recognised cellular processes, e.g. phagocytosis, release of lysozomal enzymes and K-cell cytotoxicity. It is possible that the enhancement of complement receptors may be one way through which other cellular mechanisms, which are dependent in one way or another upon the presence of complement receptors, are also enhanced.

It was observed during the course of the enhancement studies that the response to histamine was not consistent and there was a preliminary suggestion that this altered response to histamine may be related to different clinical conditions. These preliminary findings must be further substantiated by carrying our more detailed studies in which the degree of receptor enhancement is related to various disease states.

Like other effects of histamine, complement receptor enhancement may be mediated through various histamine receptors, H1- and H2-receptors. The study of these mechanisms is now possible using histamine antagonists which are known to be specific for one type of histamine receptor, i.e. either H1 or H2 antagonists. Preliminary experiments performed (not shown in the results) indicated that complement receptor enhancement may be an H1 effect since it was specifically inhibited by H1 antagonists only. However, these data are far from complete and more experimental work is needed in this area.

Experiments in which the effect of crude anaphylactic diffusates was studied for receptor enhancement activity, showed clearly that the diffusate could cause C3b receptor enhancement which was demonstrable in a dose-dependent fashion. This effect may be due not only to histamine and ECF-A tetrapeptides, but also to other high molecular fractions present in the anaphylactic diffusate. This possibility can be resolved by testing various fractions obtained from chromatographic purifications of the crude diffusate.

The studies on complement receptor enhancement will not be complete until the roles of various metabolic inhibitors in relation to this phenomenon are tested.

Studies were performed to investigate the capacity of various human leucocytes to mediate killing of schistosomula of Schistosoma mansoni in the presence of antibody alone, complement alone or in combination with antibody. The results of these studies confirmed the previous findings

of Butterworth *et al* (1975) who showed that human eosinophils could mediate damage to schistosomula coated with antibody. However, in the present study neutrophils were found to be equally effective as killer cells against antibody-coated schistosomula.

For the first time the human complement system was shown to be involved in damage of schistosomula by human leucocytes as was the ability of schistosomula to activate the alternate pathway of this system. These findings confirmed the previously reported data in which the rat eosinophil was shown to mediate killing of complement-coated schistosomula (Ramalho-Pinto *et al*, 1978). The combination of antibody and complement provided a more efficient killing mechanism which is presumably more relevant to the *in vivo* situation. The role of this system was further demonstrated by coating of the sensitized schistosomula by the purified human complement components of the classical pathway. A significant increase in the schistosomula damage was shown to be dependent upon the input of purified C3 (Fig. 26). The role of C4 and C3d in mediating C4 or C3d-coated schistosomula will need further investigation.

Preferential damage of schistosomula by human eosinophils could only be demonstrated in the presence of complement either alone or in combination with antibody (Fig. 29). The relation between the eosinophil leucocyte and the complement system with respect to schistosomula damage is unclear at the present time. It was previously suggested that the activation of the complement system at

the schistosomula surface may generate certain eosinophil chemotactic factors, probably C3a, C5a and C567 (Ramalho-Pinto et al, 1978). However, these complement-derived eosinophil chemotactic factors were also shown to be chemotactic for neutrophils as well (Ward, 1971). It was not possible for Ramalho-Pinto et al (1978) to study the role of neutrophils in compelemt-mediated damage of schistosomula since their rat eosinophil cell suspensions (obtained from peritoneal washings) contained only macrophages as contaminating cells. Therefore, preferential killing of complement-coated schistosomula by human eosinophils described in the present study is unlikely to be due to the generation of complement-derived eosinophil chemotactic factors. The most likely explanation is that adherence of eosinophils to complement-coated schistosomula may cause the cell to release cytotoxic enzymes which are quantitatively or qualitatively different from those released after the antibody-mediated adherence.

Previously the role of rat macrophages in mediating schistosomula damage was demonstrated and was shown to be dependent on IgE antibody (Capron et al, 1975). Again rat macrophages were shown to play a role during the final stages of rat eosinophil-mediated damage of schistosomula. These macrophages were shown to ingest both eosinophils which release their cytotoxic enzymes and the disrupted tegument of dead schistosomula (McLaren et al, 1978). In the present study human mononuclear cells were shown to mediate significant schistosomula damage in the presence of antibody and/or complement. No experiments were performed where various

subpopulations of the mononuclear cells were tested separately for their capacity to kill schistosomula. Such studies are important and may now be facilitated by the development of better techniques for separation of various mononuclear subpopulations.

The observation that the ECF-A tetrapeptides and histamine can enhance eosinophil complement receptors may be potentially relevant to helminth destruction *in vivo*. The IgE-mediated release of chemical mediators of anaphylaxis is well established during the early stages of contact between the host and the parasite. Therefore, one may speculate that the release of these mediators may cause eosinophils, with their enhanced complement receptors, to migrate to the site of the skin penetration where they are more efficient as killer cells (Fig. 31). Indeed, it has recently been shown that there was a significantly greater number of eosinophils at the site of cercarial challenge in the ears of immune mice (Sher, A., personal communication). Also the role of these mediators in enhancing the parasiticidal effect of rat eosinophils was recently demonstrated when the incubation of these cells with supernatants from degranulated mast cells, which presumably contained ECF-A and histamine, was shown to increase significantly the capacity of eosinophils to mediate schistosomula damage (Capron, A., personal communication). All these interesting observations will need further experimental substantiation.

In the studies described in this thesis peripheral blood eosinophils were the usual source of eosinophils. Therefore, it is possible that the results presented might

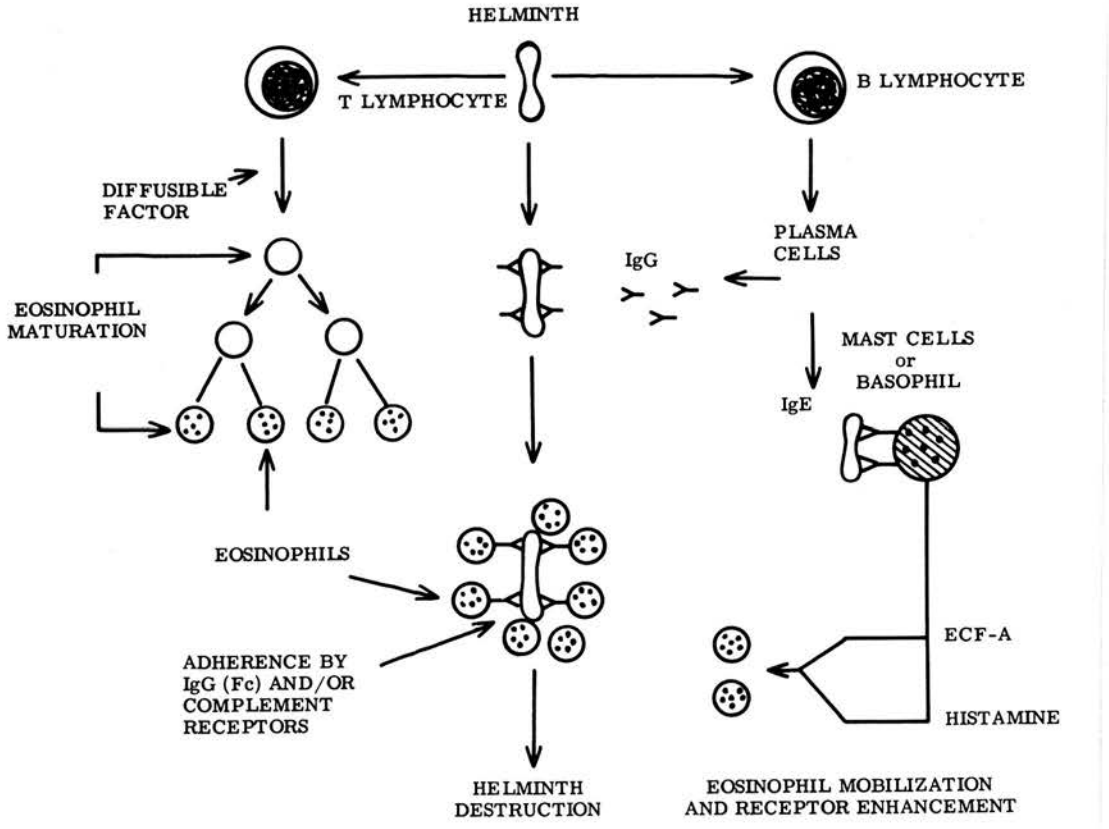

Fig. 31

Diagrammatic representation of the possible mechanisms involved in helminth destruction by eosinophils.

not be the true representation of the whole spectrum of the various eosinophil leucocyte reactions since the majority of these cells occur in the tissues rather than in the peripheral blood. However, until a convenient technique for the separation of human tissue eosinophils is available, the peripheral blood will remain the most reliable source for studies on human eosinophils.

Finally, all the studies described in this thesis were undertaken in an attempt to answer some questions in relation to the *in vivo* role of this unique cell. In this respect, the results presented here provided a number of useful insights although many more questions still need to be answered.

PUBLICATIONS

The following work connected with this thesis has either been published or submitted for publication.

ANWAR, A.R.E., KAY, A.B. (1977). Membrane surface receptors for IgG and complement (C4, C3b and C3d) on human eosinophils and neutrophils and their relation to eosinophilia.
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